University of Alberta

Testosterone Action on Gonadotropin-II Secretion from Goldfish Pituitary Cells

by

Angelina Ching-Man Lo ©

A thesis submitted to the Faculty of Graduate Studies and Research in partial fulfillment of the requirements for the degree of Master of Science

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To

my mother, Victoria

Abstract

The direct effects of testosterone (T) on gonadotropin-II (GTH-II) secretion in cultured goldfish (*Carassius auratus*) pituitary cells were investigated. Overnight pretreatment with T generally did not alter basal GTH-II secretion, but increased the salmon GTH-releasing hormone (sGnRH)- and chicken GnRH-II (cGnRH-II)-stimulated GTH-II release from pituitary cells obtained from fish at all three gonadal maturational stages (regressed, recrudescing, and prespawning). T pretreatment did not alter total pituitary cellular GTH-II contents, suggesting that T did not enhance GTH-II peptide synthesis. T selectively increased protein kinase C (PKC) activator-stimulated, but not calcium ionophore-induced, GTH-II release. Application of a PKC inhibitor abolished the GnRH-induced response in both the controls and T-treated cells. The effects of T on GnRH action were not mimicked by non-aromatizable androgens but were blocked by an aromatase inhibitor (ATD). T, *via* aromatization to estradiol, likely exerts a rapid, direct, positive influence on GnRH-stimulated GTH-II release by modulation of specific GnRH postreceptor mechanisms.

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List of Abbreviations

Abbreviations	Definitions
AA	Arachidonic acid
ATD	1, 4, 6-androstatrien-3, 17, dione
BBS	bombesin
Ca^{2+}	calcium
CAM	calmodulin
cGnRH-II	chicken GnRH-II
CCK	cholecystokinin
cDNA	complementary dioxyribonucleic acid
cAMP	cyclic adenosine triphosphates
DAG	diacylglycerol
DiC_8	1,2-dioctanoyl-*sn*-glycerol
DHT	dihydrotestosterone
DA	dopamine
E_2	17β-estradiol
FSH	follicle-stimulating hormone
GABA	γ-aminobutyric acid
GnRH	gonadotropin-releasing hormone
GSI	gonadosomatic index
GTH	gonadotropin
hCG	human chorionic gonadotropin
InsPs	inositol-1,4,5-triphosphates
11-KT	11-keto-testosterone
LH	luteinizing hormone
LHRH-A	luteinizing hormone-releasing hormone
MT	mid-tegmentum
mRNA	messenger ribonucleic acid
NE	norepinephrine
NPY	neuropeptide Y
PGF	prostaglandins $F_{2\alpha}$
PKC	protein kinase C
PLA_2	phospholipase A_2

PLC	phospholipase C
K^+	potassium
POA	preoptic area
17, 20βP	17α, 20β-progesterone
sGnRH	salmon GnRH
5HT	serotonin
TPA	phorbol-12-myristate-13-acetate
TN	terminal nerve
T	testosterone
VSCC	voltage-sensitive calcium channel

I. *Introduction*

I.1. General.

Steroid hormones are an important group of hormones produced by steroidogenic tissues. Cholesterol is the precursor in which the steroid hormones are derived from. The steroidogenic tissues of the gonads produce a number of gonadal steroids: androgens (C-19 steroids), estrogens (C-18-steroids), and progestogens (C-21 steroids). They are known to act as important regulators of reproduction. They regulate steroidogenesis in the gonads, sexual behaviour, growth, and metabolism. These effects have been extensively studied in mammals (Fink, 1988; Terasawa, 1995; Caraty *et al*, 1995).

In mammals and in other vertebrates, the control of these reproduction-related functions of gonadal steroids requires the coordinated activity of pituitary hormones which in turn are regulated by inputs from the hypothalamus. The hypothalamic gonadotropin (GTH)-releasing hormone (GnRH) regulates both luteinizing hormone (LH) and follicle-stimulating hormone (FSH) secretion from the pituitary. These pituitary GTHs act at the level of the gonads to stimulate steroid hormone production and gametogenesis. Gonadal steroid hormones and other gonadal products, such as inhibin and activin, in turn regulate the subsequent production of GTH by exerting feedback effects on the pituitary (Ying, 1988; Hadley, 1992). The regulation of this hypothalamic-pituitary-gonadal (HPG) axis is also under the control of a multifactorial system of neuroendocrine and paracrine molecules at all levels (Fink, 1988; Ying, 1988). However, feedback actions of gonadal steroids form an essential component of the multifactorial control of the HPG axis. This ensures the coordinated release of FSH and LH which is required for gonadal maturation and ovulation. Steroid

feedback is thought to involve actions at the level of hypothalamus and at the level of pituitary (Fink, 1988).

Although the reproductive success of a species depends on successful mating and rearing of the young, ovulation of matured eggs from the females must also occur at the right time for fertilization to occur. The coordination of oocyte maturation and ovulation is important for both internal and external fertilizers. In external fertilizers such as teleost fish, this coordination allows spawning to occur successfully under optimal environmental conditions. Hence, understanding the feedback effects of gonadal steroids on gonadotrope functions throughout the seasonal reproductive cycle is a crucial component in the study of reproductive control in teleost fish. This thesis reports the results of a study investigating the direct effects of testosterone (T), an androgen, on pituitary GTH release using goldfish (*Carassius auratus*) as an animal model.

In recent years, much information regarding the seasonal reproductive cycle, neuroendocrine control, and intracellular signal transduction pathways mediating pituitary GTH release in goldfish are available (Kagawa *et al*, 1983; Peter *et al*, 1990a, 1990b; Chang *et al*, 1996). The availability of these information makes goldfish a relatively good model to study the feedback actions of sex steroids on pituitary GTH release in response in teleosts. Goldfish also represents a member of the cyprinid family which contains many important commercial fish species. Information obtained from experiments with the goldfish generally is applicable to other cyprinid species. In subsequent sections, the HPG axis, seasonal reproductive cycle, as well as the positive and negative feedback actions of gonadal steroids in goldfish, will be reviewed. In addition, evidence of gonadal steroid feedback actions in other teleost and mammalian systems will be reviewed briefly.

I.2. HPG axis in teleost fish and goldfish, in particular.

As in other vertebrates, the HPG axis is involved in the neuroendocrine regulation of reproduction in fish. In the following sections, the multiple factors involved in the function and regulation of the HPG axis will be discussed with particular emphasis on the goldfish model.

I.2.1. *GTH in fish: isolation and function.*

The duality of teleost GTH has now been established in several species of teleosts. In salmonids, two GTHs, GTH-I and GTH-II, have been characterized and isolated (Suzuki *et al*, 1988b). Both in structures and functions, GTH-I resembles the tetrapod FSH while GTH-II resembles LH. These glycoprotein hormones consist of a common α-subunit and a hormone-specific β-subunit (Suzuki *et al*, 1988c). However, two very similar forms of α subunits have been identified, and GTH-I and II containing either one of these α subunit forms have been detected (Swanson, 1991). The presence of two "common" α subunits may reflect the polyploid situation in salmonids and cyprinids. The two GTH hormones have been shown to have different functions and developmental patterns. In salmonids, GTH-I and II are produced by different populations of pituitary cells. GTH-I-containing cells appear in prepubertal fish and GTH-II-containing cells appear only in postpubertal fish (Nozaki *et al*, 1990). Both GTHs are steroidogenic but their relative potencies change with gonadal development. GTH-I is thought to stimulate initiation of gametogenesis, whereas GTH-II stimulates the progression of the final stages of gonadal/germ cell maturation. Plasma GTH-I increases first at the onset of gametogenesis. Later, at ovulation and spermiation, GTH-II levels increase and GTH-I levels decrease (Suzuki *et al*, 1988a). GTH α-, GTH-I β- and GTH-II β-subunit gene expression were also shown to vary

with ovarian development (Weil *et al*, 1995). Specifically, GTH-I β mRNA levels predominate in the pituitary of previtellogenic female and prespermiating male rainbow trout (*Oncorhychus mykiss*). GTH-I β mRNA levels decrease at the start of vitellogenesis and spermatogenesis. On the other hand, a significant increase in GTH-II β mRNA levels is observed at vitellogenesis and spermatogenesis in female and male rainbow trout, respectively. There are also distinct spatial and temporal patterns of distribution for the two types of GTH receptors (GTH-R) in the gonads of coho salmon (*Onchorhynchus kisutch*) (Miwa *et al*, 1994). GTH-RI has a higher affinity for GTH-I but binds both GTHs. In contrast, GTH-RII is specific for GTH-II. GTH-RI are present in the follicular theca and granulosa cells during vitellogenesis and postvitellogenesis. GTH-RII are detected in the granulosa cells only during postvitellogenesis. The presence of two distinct GTH-R may explain why GTH-I usually is more potent than GTH-II in stimulating steroidogenesis during early gonadal development, whereas the reverse is true at later stages of gonadal maturation. Taken together, the period of early gonadal growth in the salmonids is associated with elevated plasma levels of GTH-I while the spawning period is characterized by increased plasma levels of GTH-II.

The duality of GTH form and function in teleosts is also supported by recent studies on tilapia. In this fish, GTH-II are expressed in different pituitary gonadotropes (Rosenfeld *et al*, 1996). However, unlike salmonids, there are no differences in the ontogenic appearance of GTH-I β and GTH-II β mRNA-containing pituitary cells in tilapia. Nevertheless, in the tilapia pituitary, GTH-I β mRNA levels are highest in early gonadal development while GTH-II β mRNA contents are highest in late gonadal development (Rosenfeld *et al*, 1996).

Cyprinids similarly possess two chemically distinct GTHs. But unlike salmonid species and tilapia, the functional duality of GTH-I and II, and the importance of GTH-I in stimulating early gonadal development have not been established in

cyprinid species. GTH-I and GTH-II have molecular weights of 45 kDa and 36 kDa, respectively (Habibi *et al*, 1990). These two GTHs have been purified from pituitary glands of the common carp (*Cyprinus carpio*; Van Der Kraak *et al*, 1992). These two GTHs also share a common spectrum of biological activities. Both are similarly effective in the stimulation of ovarian and testicular steroidogenesis, and induction of oocyte final maturation. Recently, the complementary deoxyribonucleic acids (cDNA) encoding the GTH α- and β-subunits have also been cloned from goldfish pituitary (Kobayashi *et al*, 1997; Yoshiura *et al*, 1997) and GTH α- and II β-subunits from carp (Chang *et al*, 1988). Two distinct genes were found to encode the GTH α-subunit. Expression of the GTH α-subunits in the goldfish pituitary does not vary with gonadal maturity (Kobayashi *et al*. 1997). On the contrary, expression of the two types of cDNAs that encode the β-subunit of goldfish GTH varies with differing stages of ovarian maturity. Highest levels of both GTH-I and II β-subunit mRNAs were observed during ovarian maturity and lowest levels of both were detected in the regressed stage. These results suggest that the synthesis of both types of GTHs are similarly affected by gonadal development (Yoshiura *et al*, 1997). Whether GTH-I and GTH-II are produced by different pituitary cells in the common carp and goldfish is unknown. More information on the secretion and the regulation of GTH-I in goldfish is required. In contrast, much more is known about the neuroendocrine regulation of GTH-II in goldfish. This thesis is restricted to studies on GTH-II secretion only.

The duality of GTHs is well documented in some but not all families of teleosts. The African catfish, *Clarias gariepinus*, may express only GTH-II-like hormone in the pituitary (Schulz *et al*, 1995). GTH-II content in pituitary extract accounts for all of the extract's steroidogenic activity. In addition, all gonadotropes contain GTH-II β-subunit immunoreactivity and GTH-I β-gene appears to be absent in these cells (Schulz *et al*, 1995). GTH-I-like hormone might not be expressed in the African catfish pituitary. European eel (*Auguilla auguilla*) is another teleost species in

which GTH-I-like hormones could not be isolated or detected (Quérat, 1995). Thus, although the duality of teleostean GTHs is well-established, it may not apply to all teleosts. At present, the neuroendocrine control of GTH-II secretion and synthesis in fish is better studied, and more is known about its regulation than that for GTH-I.

I.2.2. *Hypothalamic regulators of GTH-II release in goldfish.*

Unlike most other vertebrates, the equivalent of the median eminence is located in the adenohypophysis in teleosts. As a result, in goldfish, many of the hypothalamic factors involved in the regulation of GTH-II release from the pituitary are delivered directly by neurosecretory fibres to the vicinity of the gonadotropes where they may directly or indirectly alter GTH-II secretion (Peter *et al*, 1990a).

In goldfish, as in other vertebrates, GnRH is an important stimulatory hypothalamic factor for GTH-II release. GnRH nerve terminals are located in the proximal pars distalis where gonadotropes are found (Kah *et al*, 1986a). Other evidence also indicates a direct action of GnRH on GTH-II release (Chang *et al*, 1990a). Other hypothalamic factors known to stimulate GTH-II release include neuropeptide Y (NPY), γ-aminobutyric acid (GABA), bombesin (BBS), cholecystokinin (CCK), norepinephrine (NE), and serotonin (5HT). The GTH-II stimulatory action of NPY, NE, and 5HT have been shown to be direct, at the level of the pituitary cells, as well as indirect, by increasing GnRH release (Chang and Peter, 1984; Somoza *et al*, 1988; Peng *et al*, 1990, 1993a; Somoza and Peter, 1991; Yu *et al*, 1991; Chang *et al*, 1991b; Sloley *et al*, 1992; Wong *et al*, 1993). GABA has been shown to stimulate GTH-II secretion solely by altering GnRH secretion and not by direct action on the gonadotropes (Kah *et al*, 1992). On the other hand, the effects of BBS and CCK on GTH-II release have only been examined in pituitary fragments *in vitro* (Himick and Peter, 1995; Himick *et al*, 1996). Thus, whether BBS and CCK exert their effects

directly on gonadotropes, or indirectly through influences on other neuroendocrine factors, is still unknown. In addition to regulation by stimulatory factors, GTH-II release in goldfish is under the direct inhibitory influence of dopamine (DA; Kah *et al*, 1986a, 1986b; Peter *et al*, 1990b). DA has been shown to directly inhibit basal GTH-II secretion, as well as GnRH-, NPY-, and 5HT-stimulated GTH-II secretion. Among these neuroendocrine factors, innervation of the pituitary by DA-, NPY-, CCK-, BBS-, and GABA-containing hypothalamic nerve fibres have been confirmed (Kah *et al*, 1984, 1992, 1993; Himick and Peter, 1995; Himick *et al*, 1996). However, because of the lack of evidence for direct innervation of the goldfish pituitary by 5HT- and NE-containing hypothalamic neurons, the direct GTH-II releasing actions of 5HT and NE may be from local paracrine and peripheral sources, respectively (Chang *et al*, 1991a; Kah *et al*, 1993; Wong, 1993).

Of these multiple hypothalamic regulators of GTH-II release in goldfish, GnRH and DA appear to be some of the most important factors (Peter *et al*, 1986, 1991). As a result, the GnRH and DA systems in goldfish and their relationship to GTH-II secretion will be discussed in more detail below.

I.2.2.1. GnRH system in goldfish.

Multiple GnRH forms are present in the goldfish as in other teleosts and vertebrates (see Powell *et al*, 1994; Sower *et al*, 1995; King and Millar, 1995 for reviews of multiple GnRH forms in vertebrates). In goldfish, the presence and regional distribution of two endogenous GnRH forms, [Trp^7, Leu^8]-GnRH (salmon GnRH, sGnRH) and [His^5, Trp^7, Tyr^8]-GnRH (chicken GnRH-II, cGnRH-II), in the brain have been characterized by a combination of high pressure liquid chromatography and radioimmunoassay (Yu *et al*, 1987, 1988; Rosenblum *et al*, 1994). Recently, cDNA sequences encoding the precursor for these two GnRHs have also been cloned from

goldfish and the mRNA for both GnRHs have been detected in goldfish brain tissues (Lin and Peter, 1996). These findings confirm the presence of these two GnRH forms in this species.

Neuronal cell bodies containing GnRH are found in the telencephalon-preoptic area (T-POA), the midbrain-tegmentum (MT), and the terminal nerve (TN) (Kah *et al*, 1993). sGnRH is present in the TN and T-POA system while cGnRH-II is present in the POA and the MT. Nerve fibres from the POA and MT project to the adenohypophysis (Kah *et al*, 1986a) and both sGnRH- and cGnRH-II-containing nerve terminals have been detected in the pituitary (Kim *et al*, 1995). In other vertebrates, the MT-GnRH system is usually thought to be associated with the regulation of sexual behaviour rather than the regulation of GTH-II release (Yu *et al*, 1997). Thus, whether pituitary cGnRH-II nerve terminals are derived from the POA system, MT system, or both systems in the goldfish is not clear. It is generally agreed that the POA-GnRH system regulates pituitary GTH-II secretion (Yu *et al*, 1997). Since both sGnRH and cGnRH-II are found in this brain area, as well as the pituitary, both GnRHs likely play a role in GTH-II release in goldfish. Although GnRH from the TN contributes to the majority of the sGnRH contents in the brain, this neuronal system does not contribute to pituitary GnRH content (Kobayashi *et al*, 1994). Furthermore, abolition of the TN-sGnRH system does not affect ovulation in the goldfish. Taken together, these data indicate that the TN-sGnRH system does not control pituitary GTH-II secretion.

Not only are sGnRH and cGnRH-II nerve terminals present in the goldfish pituitary, both GnRHs can also be released from pituitary fragments *in vitro* (Yu *et al*, 1991). This suggests that these two GnRHs may be released at the level of the pituitary *in vivo*. The ability of sGnRH, cGnRH-II, as well as other naturally occurring GnRH forms and their analogs, to induce GTH-II release in goldfish has been demonstrated *in vivo*, and in *in vitro* experiments with pituitary fragments and dispersed pituitary cells (Habibi *et al*, 1989b; Chang *et al*, 1990a). Conversely,

application of an antagonistic GnRH analog ([Ac-Δ^3-Pro^1, 4FD-Phe^2, D-Trp^{3,6}]-mGnRH, analog E) suppressed sGnRH- and cGnRH-II-stimulated GTH-II release from pituitary fragments and cells *in vitro*, and reduced basal and GnRH-induced increases in serum GTH-II levels *in vivo* (Murthy *et al*, 1994). A decrease in brain GnRH content has also been shown to precede the increase in GTH-II release that accompanies ovulation and spawning (Yu *et al*, 1987). Taken together, these data indicate that both GnRH forms are physiological regulators of GTH-II release in the goldfish.

Although both sGnRH and cGnRH-II are able to regulate GTH-II release in goldfish, whether they serve different functions has not been directly studied. Recently, Rosenblum *et al*. (1994) reported that the brain sGnRH and cGnRH-II contents in the T-POA and pituitary were higher in adult than in prepubertal (juvenile) goldfish. The magnitude of K^+ (depolarization)-induced GnRH release is greater from pituitary fragments obtained from adult fish than juvenile fish. However, this pubertal-dependent increase in sGnRH content and inducible release is greater than those for cGnRH-II. It is possible that sGnRH is a major stimulator of pubertal maturation although both GnRH forms participate in the neuroendocrine regulation of GTH-II secretion.

I.2.2.2. DA : an inhibitory regulator in goldfish.

GTH-II secretion in goldfish is under tonic inhibitory control by DA. Hypothalamic dopaminergic neurons, originating from the anteroventral preoptic region, innervate the proximal pars distalis (Peter *et al*, 1990b). DA released from these nerve terminals directly inhibits basal GTH-II secretion, both *in vivo* and *in vitro* (Chang and Peter, 1983; Peter *et al*, 1986). DA also inhibits GnRH-stimulated GTH-II secretion from pituitary by activating DA-D_2 receptors (Chang *et al*, 1993). In addition,

stimulation of DA-D_2 receptors also decreases the number of GnRH receptors in the goldfish pituitary (de Leeuw *et al*, 1989). DA also inhibits GnRH release *in vitro* from preoptic-anterior hypothalamic brain slices via D_1 receptors, and GnRH release from pituitary fragments *via* D_2 receptors (Yu *et al*, 1991, 1997). The close anatomical relationship between the DA and GnRH neurons also provides evidence for the possible interactions of these two systems at both the hypothalamus and pituitary levels in goldfish (Kah *et al*, 1986a). These interactions of the stimulatory and inhibitory systems ensure that GTH-II secretion is tightly regulated.

I.2.3. *Other local factors regulating pituitary GTH-II release in goldfish.*

In addition to hypothalamic factors, other paracrine factors also affect GTH-II secretion from the pituitary. As discussed briefly in the previous section on hypothalamic factors (section I.2.2), a paracrine, stimulatory 5HT influence has been proposed. 5HT is present in pituitary cells and not in nerve terminals in the goldfish. The source of this 5HT may be from local production or uptake from peripheral sources (Kah *et al*, 1993). Aside from 5HT, activin and inhibin are also possible paracrine regulators of GTH-II secretion. Activin and inhibin subunits are present in the goldfish pituitary cells. Application of either activin or inhibin stimulates basal GTH-II secretion from dispersed goldfish pituitary cells and enhances GnRH-induced GTH-II response (Ge *et al*, 1992).

I.2.4. *Regulation of gonadal steroid production and gamete maturation in goldfish.*

Gonadal steroid hormone biosynthesis is regulated primarily by GTH-I and II. Other factors such as prolactin, growth hormone, and locally-produced factors including insulin-like growth factor could also be involved (Singh *et al*, 1988; Van Der Kraak *et al*, 1990; Srivastava *et al*, 1994). In goldfish, developing oocytes are surrounded by two major follicular cell layers, an outer thecal cell layer and an inner granulosa cell layer, separated from each other by a basement membrane. These two cell layers act in a cooperative fashion in estrogen biosynthesis in response to GTH stimulation. The thecal layer is the major site of biosynthesis of androgens from cholesterol; androgens (androstenedione and/or T) are then transported to the granulosa layer and aromatized to 17β-estradiol (E_2; Nagahama, 1994, 1995). GTH-I and II have similar effects on steroid production in previtellogenic and vitellogenic follicles. In contrast, GTH-II is more potent than GTH-I in stimulating T and E_2 production from goldfish preovulatory postvitellogenic follicles *in vitro* (Van Der Kraak *et al*, 1992). Gonadal steroids play an important role in the maturation of ovarian follicles and oocytes. During vitellogenesis, E_2 has been shown to induce the synthesis and secretion of vitellogenin from the liver, and its uptake into oocytes. Following vitellogenesis, final oocyte maturation and ovulation is also under the influence of GTH-stimulated steroid production. In response to GTH (possibly GTH-II), 17α, 20β-dihydroxy-4-pregnen-3-one (17, 20βP) is produced and this steroid stimulates final oocyte maturation. 17, 20βP induces the oocytes to synthesize cyclin B, which in turn activates and produces cdc2 kinase. These factors are thought to be responsible for the initiation of nuclear membrane breakdown and cell division during germinal vesicle breakdown in the final oocyte maturation process (Nagahama, 1994). As in the case for E_2 production, both follicular cell types are involved in 17, 20βP production. The thecal cell layer produces 17α-hydroxyprogesterone. This traverses the basal lamina and is converted to 17, 20βP in the granulosa cell layer where GTH acts to enhance the activity of 20β-hydroxysteroid dehydrogenase (Nagahama, 1990).

As in females, two gonadal cell types play a role in coordinating the action of GTH on the testes of male goldfish. In the testis, the interstitial cells are a major source of androgen synthesis. Under the stimulation of GTH, T and 11-ketotestosterone (11-KT) are produced. Although both GTH-I and II stimulate androgen production from testicular fragments *in vitro*, GTH-II is more potent than GTH-I (Van Der Kraak *et al*, 1992). T and 11-KT are involved in spermatogenesis, and in the development of male secondary sexual characteristics (Kobayashi *et al*, 1991). The action of 11-KT on spermatogenesis is mediated by its action on the Sertoli cells. 11-KT stimulates the Sertoli cells to produce activin B, which then acts on spermatogonia to induce mitosis leading to the formation of spermatocytes (Nagahama, 1994). During spermatogenesis, plasma levels of T and 11-KT are high, but these levels decline after the onset of spermiation. At final testicular maturation, there is a switch from a predominance of 11-oxygenated androgens to a production of progestogens, including 17, 20βP (Abdullah and Kime, 1994). 17, 20βP mediates the stimulatory effects of GTH-II on spermiation (Zheng and Stacey, 1997).

I.2.5. *Gonadal steroid feedback on GTH-II secretion in goldfish.*

The production of gonadal steroids is also regulated by feedback actions of these steroids on the HPG axis. Both negative and positive feedback effects are detected in goldfish.

I.2.5.1. Negative feedback effects of gonadal steroids.

Earlier studies in goldfish showed that implantation of anti-estrogen into the hypothalamus and pituitary caused ovulation in sexually mature fish (Peter, 1983). This suggests that negative feedback effects of gonadal steroids exist. The presence of

negative feedback actions of gonadal steroids was also demonstrated by gonadectomy experiments. Removal of the ovaries (ovariectomy) from sexually mature female goldfish elevated blood GTH-II levels; the subsequent implantation of T and E_2 depressed these elevated GTH levels in plasma (Kobayashi and Stacey, 1990). However, the pituitary GTH-II contents did not differ in these ovariectomized female goldfish (Kobayashi and Stacey, 1990). This indicates the existence of negative feedback by T and E_2 on basal GTH-II release, but not GTH-II synthesis, in the female goldfish. In contrast, 11-KT does not play a role in the negative feedback effects of gonadal steroids in females (Kobayashi *et al,* 1991).

In the male goldfish, negative effects of E_2 on androgen synthesis have been shown by E_2 implantation (Trudeau *et al*, 1993f). Following E_2 treatment, basal testicular production of T and 11-KT were reduced, but circulating GTH-II levels were not affected. E_2 production at the level of the testes could be exerting a direct inhibitory effect on the release of androgens without altering pituitary GTH-II release in male goldfish.

Taken together, these observations indicate that negative steroid feedback effects can be manifested as a change in GTH-II release, as well as inhibitory actions directly at the level of the gonads.

I.2.5.2. Positive feedback effects of gonadal steroids.

The positive feedback effects of gonadal steroids on GTH-II release from pituitary of gonad-intact goldfish has been demonstrated by Trudeau *et al* (1991b). Intraperitoneal implantations of T and E_2 capsules into gonad-intact female and male goldfish for 5 days potentiated mammalian GnRH (LHRH) analogue [D-Ala^6, Pro^9]-*N*-ethylamide-LHRH; LHRH-A)-stimulated increase in serum GTH-II. T potentiates the LHRH-A-induced GTH-II release throughout the seasonal reproductive

cycle; the potentiating effect of E_2, however, is more restricted to sexually regressed goldfish.

This positive feedback effect of T on inducible GTH-II release is likely mediated by aromatization of T to E_2. The implantation of aromatase inhibitor (1,4,6-androstatrien-3, 17-dione; ATD) inhibited the ability of T to enhance the LHRH-A-induced GTH-II secretion (Trudeau *et al*, 1991b). On the other hand, implantations of either 11-KT or DHT into female fish did not affect LHRH-A-induced GTH-II release. Thus, T, through aromatization to E_2, increases the pituitary GTH-II release-responsiveness to LHRH-A stimulation.

The positive feedback effect of steroids on the *in vitro* release of GTH-II from pituitary fragments also has been demonstrated. *In vivo* implantation of T or E_2 for 5 days potentiated sGnRH-, cGnRH-II-, and sGnRH-agonist-stimulated GTH-II secretion from goldfish pituitary fragments *in vitro* (Trudeau *et al*, 1993a). These positive effects of steroids were not mediated by changes in the pituitary GTH-II contents, GnRH receptor affinity, GnRH receptor number, or the responsiveness to DA-D_2 inhibition. *In vitro* exposure of pituitary fragments to T for 24 hours also enhanced the GTH-II response to sGnRH. Coincubation with cycloheximide (protein synthesis inhibitor) abolished this enhanced GTH-II response by T, suggesting that protein synthesis is involved (Trudeau *et al*, 1993a).

I.2.5.3. Sites of steroid feedback and steroid action.

As discussed in the sections I.2.5.1 and I.2.5.2, both negative and positive feedback effects of gonadal steroids on GTH-II release have been observed in female and male goldfish. Anatomical localization of gonadal steroid receptors and steroid metabolizing enzymes provide evidence that the sites of these feedback actions are at the levels of the hypothalamic neurons and pituitary. Earlier studies have

demonstrated the presence of aromatase and 5α-reductase activities in the pituitary and several brain regions including telencephalic, preoptic, and hypothalamic areas (Kim *et al*, 1978; Pasmanik and Callard, 1988a). The brain distribution of E_2 binding sites also roughly corresponds to that for aromatase activity. These forebrain regions are known to be important hypophysiotropic areas. A high abundance of androgen receptors has also been observed in goldfish forebrain and pituitary (Pasmanik and Callard, 1988b; Gelinas and Callard, 1997). The presence of steroid metabolizing enzymes in brain and pituitary indicates that circulating gonadal steroids can either act by themselves, or may be converted to "local active" forms prior to exerting their action. The presence of aromatase in the higher levels of the HPG axis further suggests that local aromatization of T to E_2 plays a role in the feedback action of T on GTH-II secretion. The detection of androgen receptors in the brain and pituitary indicates that T and its nonaromatizable metabolites may also act directly at these two levels of the HPG axis.

Although GnRH provides a major stimulatory influence on GTH-II release, steroid feedback actions are not mediated by direct actions on GnRH neurons. Steroid receptors have not been localized on GnRH neurons (Kah *et al*, 1993). Recent evidence indicates that steroid feedback may affect the GnRH system through actions on other neuroendocrine regulators, including DA, GABA, NPY, and NE.

Four lines of evidence indicate that gonadal steroids affect GTH-II release by modulating brain and pituitary DA levels. 1) Anatomical data suggest that gonadal steroids can influence the activity of DA neurons. Aromatase activity and steroid receptors are present in the POA-anterior hypothalamic region of goldfish where dopamine neuronal cell bodies are located (Kah *et al*, 1986b; Pasmanik and Callard, 1988a, b; Gelinas and Callard, 1997). 2) There is direct evidence that steroid treatments alter DA turnover in the brain and pituitary. Implantation of T in sexually regressed goldfish decreased DA turnover in the POA and hypothalamus, but increased DA turnover in the pituitary. In fish undergoing gonadal recrudescence, T and E_2 treatment

increased DA turnover in the POA. Similarly, T-treatment increased pituitary DA turnover in recrudescing goldfish (Trudeau *et al,* 1993b). 3) A functional link between DA turnover and GTH-II release has been established. Exposure of male goldfish to female pheromone 17, 20β P resulted in an increase in GTH-II release. This release in GTH-II is associated with a decrease in DA turnover in the pituitary (Dulka *et al,* 1992). This suggests that a decreased DA release from nerve terminals in the pituitary mediates increases in GTH-II secretion under certain physiological conditions. 4) The seasonal increase in the inhibitory DA tone on circulating GTH-II levels with gonadal maturation (Sokolowska *et al*, 1985; Sloley *et al*, 1991) is positively correlated with increased circulating steroid levels and the ability of steroid to increase DA turnover at the level of the pituitary. As indicated in section I.2.2.2 above, DA can alter GnRH neuronal activity at both the POA-anterior hypothalamic and pituitary levels. Taken together, these data strongly suggest that steroidal influence on GTH-II release can be exerted through DA neurons and their direct effects on pituitary GTH-II secretion, as well as through DA's actions on the GnRH system.

Steroid feedback control of GTH-II secretion may also be mediated by steroid actions on other hypothalamic neuronal systems such as GABA. The brain distribution of GABA immunoreactive fibres and androgen receptors overlap one another, especially in POA and the medial basal hypothalamic areas including the nucleus lateralis tuberis (Kah *et al*, 1993; Gelinas and Callard, 1997). The nucleus lateralis tuberis is one of the origins of GABA neurons innervating the pituitary (Kah *et al*, 1993). GABA synthesis rates in the POA and pituitary were increased by T or P_4 implantation, while pituitary and hypothalamus GABA synthesis rates were elevated by E_2 (Kah *et al*, 1992). GABA has no direct effect on GTH-II release, but it has been shown to indirectly increase GTH-II secretion by stimulating GnRH release at the level of the pitutiary (Kah *et al*, 1992). GABA may also indirectly affect GTH-II secretion by decreasing pituitary and hypothalamic DA turnover rates (Trudeau *et al,* 1993b, 1993c).

Implantation of E_2 abolished the ability of GABA to increase GTH-II release in fish in sexually regressed and early recrudescence stages (Kah *et al*, 1992). In contrast, T implantations enhanced the stimulatory effect of GABA on serum GTH-II levels in sexually regressed goldfish and fish at late stages of recrudescence (Kah *et al*, 1992; Trudeau *et al*, 1993d). These results suggest that steroids affect GABA neuronal activity, and this in turn alters the activity of the DA and GnRH systems. In addition, gonadal steroids also modulate the stimulatory effects of GABA on GnRH secretion; the positive effects of T may be mediated in part by modulating the ability of GABA to increase GnRH secretion.

As with DA and GABA, NPY neurons may also be a target of steroid feedback action in goldfish. NPY perikarya are located in the caudal ventral telencephalic area and POA (the nucleus preopticus and nucleus preopticus periventricularis; Kah *et al*, 1993), areas that have been shown to contain aromatase and androgen receptors (Gelinas and Callard, 1997). Implantation of E_2 or T increased NPY mRNA expression, especially in the ventral telencephalic brain areas (Peng *et al*, 1994). These telencephalic areas have been proposed to be involved in the expression of olfactory mediated phermonal effects on GTH-II release and sexual behaviour (Kyle *et al*, 1982). In addition, treatment with these steroids increased the stimulatory effects of NPY on GnRH release *in vivo*, but not NPY-induced GTH-II release *in vitro* (Peng *et al*, 1993b). These results indicate that the feedback effects of gonadal steroids on GTH-II release are likely to include an NPY-dependent component at the level of higher brain centers rather than at the level of the pituitary.

The brain NE neuronal system has similarly been implicated as a possible mediator of steroid feedback action. Implantation of T decreased NE turnover in the POA in fish undergoing sexual recrudescence. On the other hand, E_2 decreased NE turnover in the POA and hypothalamus in sexually regressed fish, but increased NE turnover in these brain areas in recrudescent fish (Trudeau *et al*, 1993e). As indicated in

an earlier section (I.2.2), NE stimulates GnRH secretion from POA-anterior hypothalamic slices. In addition, recent results indicate that blockade of GABA degradation decreased NE turnover in the POA (Trudeau *et al,* 1993e). Taken together, it is likely that steroid feedback also alters GnRH neuronal activity via modulation of NE neuronal function in the POA area. The action of steroid on NE may be indirect, through alteration of GABA neuronal activity.

Although the information is scant, steroid feedback action on GTH-II release may also involve modulation of the taurine (TAU) system. T and E_2 implantation potentiated the GTH-II releasing effects of TAU injection (Trudeau *et al,* 1993d). Whether steroid treatment affects brain and pituitary TAU levels or its metabolism has not been demonstrated and the physiological role of TAU in the neuroendocrine regulation of GTH-II has not been established.

Besides action on higher brain centers, steroid effects on GTH-II release may also be exerted directly at the level of the pituitary via alteration of GTH-II production. The effects of T on GTH-II β-subunit mRNA levels have been demonstrated in goldfish pituitary (Huggard *et al,* 1996). Injections of T or the nonaromatizable androgen, 11β-hydroxyandrosterone, into sexually immature goldfish resulted in an initial decrease in GTH-II subunit mRNA levels. This is followed by an increase in GTH-II subunit mRNA levels after a longer treatment time. In studies on the dose-dependent effects of sex steroids, injections with lower doses (0.2 and 2 μg/fish) of T for 24 hours resulted in a stimulation, while a higher dose (20 μg/fish) caused a decrease in GTH-II subunit mRNA production in immature goldfish. In mature (prespawning) goldfish, no such biphasic effect of T was observed and the GTH-II mRNA levels increased with all doses of T injected *in vivo*. *In vitro* treatment of pituitary fragments obtained from sexually immature and mature goldfish with doses of T ranging from 2 to 500 ng/ml for 15 hours increased GTH-II α- and β-subunit mRNA

levels. These results demonstrate that T is effective in stimulating basal GTH-II subunit mRNA production in the goldfish pituitary.

As discussed in section I.2.5.1, sex steroids can also exert local actions at the level of the gonads.

I.3. Transduction mechanisms mediating GnRH action on GTH-II release in goldfish.

The signal transduction mechanisms mediating GnRH action on GTH-II secretion in goldfish have been studied in some detail and will be briefly reviewed below.

I.3.1. *GnRH receptors.*

High affinity/low capacity and low affinity/high capacity GnRH binding sites have been detected in goldfish pituitary membrane preparations by radioligand binding assays (Habibi *et al*, 1989b). However, photoaffinity labelling revealed the presence of two high affinity GnRH receptors with slightly different molecular weights. sGnRH- and cGnRH-II-stimulated GTH-II release are correlated with binding to the high affinity sites (Habibi *et al*, 1989b). Whether sGnRH and cGnRH-II stimulate GTH-II release *via* separate GnRH receptors is still unclear. It has been proposed that the two high affinity GnRH receptor forms revealed by photoaffinity labelling reflect GnRH receptors on gonadotropes and somatotropes, respectively (Habibi *et al*, 1990).

Six lines of evidence from past studies have demonstrated that sGnRH and cGnRH-II can act on the same class of GnRH receptors to stimulate GTH-II release. 1) sGnRH and cGnRH-II displace radio-labelled GnRH agonist from pituitary

membrane preparations with equal potency and effectiveness (Murthy *et al*, 1994). 2) sGnRH and cGnRH-II also displace avidin gold-labelled sGnRH-analog binding to immunohistochemically identified GTH-II cells *in vitro* (Cook *et al*, 1991). 3) The GTH-II responses to maximal effective doses of sGnRH and cGnRH-II were not additive (Chang *et al*, 1993). 4) There is a cross-desensitization of GTH-II response by either GnRHs. Further stimulation by sGnRH or cGnRH-II is inhibited after a prolonged treatment of either GnRHs (Murthy and Peter, 1994). 5) sGnRH- and cGnRH-II-induced GTH-II release were equally inhibited by competitive GnRH antagonists based either on sGnRH or cGnRH-II primary sequence (Murthy and Peter, 1994). 6) Sequential applications of sGnRH and cGnRH-II increase the intracellular calcium levels ($[Ca^{2+}]_i$) in the same identified gonadotrope (Van Goor, 1997). However, other studies indicate the involvement of different receptors for sGnRH and cGnRH-II. Khakoo *et al* (1994) showed a lower degree of desensitizaton in GTH-II response to continuous heterologous (sGnRH/cGnRH-II or cGnRH-II/sGnRH) treatments compared with homologous (sGnRH/sGnRH or cGnRH-II/cGnRH-II) treatments. The signal transduction mechanisms mediated by sGnRH and cGnRH-II on GTH release were also shown to differ (Chang *et al*, 1996; also see sections I.3.2, I.3.3, and I.3.6 below). These results suggest the presence of different GnRH receptors with partially overlapping GnRH binding/activation properties. However, slight differences in homologous versus heterologous GnRH-induced desensitization, as well as the dissimilarity in signal transduction, can also be the result of differential receptor-effector coupling of the same receptors by the two native GnRHs.

Recently, a GnRH receptor that can bind to and can be activated equally by both sGnRH and cGnRH-II has been cloned from goldfish brain and pituitary cDNA library (He *et al*, 1997). This suggests that a common sGnRH and cGnRH-II receptor exist in the goldfish although the presence of other GnRH receptors cannot be excluded. This goldfish GnRH receptor has a seven-transmembrane structure typical

for other cloned GnRH receptors and guanine nucleotide binding protein (G-protein)-coupled receptors (Stojilkovic *et al*, 1994; Tensen *et al*, 1997). G-proteins play a major role in many receptor-effector coupling systems, linking the receptor with various intracellular signal transduction effector systems such as phospholipase C (PLC), PLA_2, and adenylate cyclase. In goldfish, G-protein coupling in GnRH action has not been directly studied. However, the ability of pertussis toxin and cholera toxin to increase GTH-II release suggests that G-proteins are present in the goldfish gonadotropes (Chang *et al*, 1993, 1996).

I.3.2. *Phospholipase C and inositol phosphates.*

PLC action generates inositol phosphates (InsPs) and diacylglycerol (DAG). Ins-1,4,5-P_3 binding to its receptors causes mobilization of intracellular Ca^{2+} from nonmitochondrial stores while DAG activates protein kinase C (PKC) (see review by Berridge, 1987). Both sGnRH and cGnRH-II actions have been linked to activation of PLC in the goldfish. Incubation of goldfish pituitary cells with sGnRH stimulated the production of $InsP_1$, $InsP_2$, $InsP_3$ and other higher InsPs; similar treatment with cGnRH-II increased the production of only $InsP_2$ (Chang *et al*, 1995). Thus, cGnRH-II may be activating different PLC isoforms and/or different InsPs metabolizing enzymes, which then leads to the formation of different InsPs as compared to sGnRH.

I.3.3. *Ca^{2+}.*

The involvement of Ca^{2+} in mediating sGnRH- and cGnRH-II-induced GTH-II release has been demonstrated (reviewed in Chang *et al*, 1996). Briefly, removal of extracellular Ca^{2+} or the blockade of extracellular Ca^{2+} entry attentuated the GTH-II responses to sGnRH and cGnRH-II in both short-term release (perifusion)

studies, as well as in long-term (2 h) static incubation experiments. The pharmacological profile of the inhibitors of Ca^{2+} entry used suggests that extracellular Ca^{2+} entry through voltage-dependent Ca^{2+} channels (VSCC), and the subsequent increase in $[Ca^{2+}]_i$ is involved in GnRH action. The ability of the VSCC agonist Bay K 8644 to enhance GnRH-induced GTH-II release, as well as the identification of such channels in identified goldfish gonadotropes (Van Goor *et al,* 1996), are consistent with this hypothesis. However, cGnRH-II action on GTH-II release is more sensitive to manipulation of extracellular Ca^{2+} availability and VSCC activity than that for sGnRH. This suggests that cGnRH-II action is relatively more dependent on the availability of extracellular Ca^{2+} than is sGnRH action. Since sGnRH, but not cGnRH-II, causes $InsP_3$ production (Chang *et al*, 1995), it is possible that sGnRH action on gonadotropes has a comparatively greater intracellular Ca^{2+} mobilization component than cGnRH-II.

The ability of sGnRH and cGnRH-II to increase $[Ca^{2+}]_i$ has been confirmed in mixed populations of dispersed goldfish pituitary cells (Jobin and Chang, 1992a) and in identified gonadotropes preloaded with Ca^{2+}-sensitive dyes (Mollard and Kah, 1996; Van Goor, 1997). The ability of the GnRH antagonist, analog E, to block these responses in identified gonadotropes indicates the involvement of GnRH receptor binding in mediating these $[Ca^{2+}]_i$ responses (Van Goor, 1997). However, the relative contribution of Ca^{2+} from both extracellular and intracellular Ca^{2+} sources to the GnRH-induced Ca^{2+} response is still controversial. Prior incubation with Ca^{2+}-deficient medium abolished the cGnRH-II-stimulated $[Ca^{2+}]_i$ responses and partially reduced the sGnRH-induced increase in $[Ca^{2+}]_i$ in mixed population of dispersed goldfish pituitary cells (Jobin and Chang, 1992a). This is consistent with the proposed differential dependence of sGnRH- and cGnRH-II-stimulated Ca^{2+} responses (Jobin and Chang, 1992a). However, in one study with identified goldfish gonadotropes, the ability of sGnRH and cGnRH-II to increase $[Ca^{2+}]_i$ were both abolished by removal of

extracellular Ca^{2+} (Mollard and Kah, 1996). This suggests that the actions of both native GnRHs are completely dependent on extracellular Ca^{2+}. The reasons for these discrepancies are not known and differences in experimental protocols do not facilitate the comparison of results. The intracellular free Ca^{2+} level is affected by many factors and the balance between many calcium stores. Prolonged withdrawal of extracellular Ca^{2+} availability may affect intracellular stores. Clarification of the relative dependence of extracellular and intracellular Ca^{2+} in GnRH action requires further detailed characterization of Ca^{2+} homeostasis. Despite these ambiquities in results with $[Ca^{2+}]_i$ measurements, extracellular Ca^{2+} availability appears to be an important factor in GnRH-induced GTH-II release. Regardless of the roles of intracellular and extracellular Ca^{2+} in immediate GTH-II release, extracellular Ca^{2+} is probably required for prolonged GnRH stimulation of GTH-II secretion (Jobin *et al*, 1992b).

I.3.4. *PKC*.

Both sGnRH and cGnRH-II action on GTH-II release have been shown to be mediated by PKC. As discussed above (section I.3.2), GnRH stimulates PLC-mediated hydrolysis of phosphoinositides which generates DAG, an endogenous activator of PKC. Addition of drugs that stimulate PKC such as tumor-promoting 4β-phorbol esters, including tetradecanoyl phorbol 13 acetate (TPA), or a synthetic DAG, dioctanoylglycerol (DiC_8), increased GTH-II secretion from goldfish pituitary cells (Chang *et al*, 1991a). PKC inhibitors, on the other hand, reduced the GTH-II release stimulated by PKC activators, sGnRH and cGnRH-II (Chang *et al*, 1991a; Jobin *et al*, 1996b). In PKC-depleted cells, GTH-II responses to sGnRH, cGnRH-II, and a superactive sGnRH-analogue were significantly reduced (Jobin and Chang, 1993; Jobin *et al*, 1993).

PKC likely mediates GnRH action on Ca^{2+} mobilization and extracellular Ca^{2+} entry through VSCC. Treatment with Ca^{2+}-deficient medium or additions of VSCC inhibitors reduced TPA- and DiC8-stimulated GTH-II release (Chang *et al*, 1991a; Jobin and Chang, 1992a). Conversely, application of Bay K 8644 or depolarizing doses of K^+ enhanced TPA-induced GTH-II responses (Jobin *et al*, 1996b). An extracellular Ca^{2+} dependence is also evident in the TPA- and DiC8-stimulated increase in $[Ca^{2+}]_i$ in dispersed goldfish pituitary cells (Jobin and Chang, 1992a). Moreover, application of a PKC inhibitor reduced the $[Ca^{2+}]_i$ responses to TPA and cGnRH-II in mixed populations of dispersed goldfish pituitary cells (Jobin and Chang, 1992a). Taken together, these data strongly indicate that PKC mediates the GnRH activation of VSCC and that the subsequent increase in $[Ca^{2+}]_i$ may potentiate the GTH-II-releasing action of PKC-dependent signalling components. However, how PKC activates VSCC is at present unknown. Application of TPA did not increase voltage-dependent Ca^{2+} currents in preliminary patch-clamp studies with identified goldfish gonadotropes (Van Goor, 1997).

I.3.5. *Calmodulin.*

Besides PKC, another possible site of action for elevated levels of $[Ca^{2+}]_i$ in cytosol is calmodulin (CaM). The addition of calmidazolium, a CaM antagonist, or KN62, a Ca^{2+}/CaM-dependent protein kinase II inhibitor, reduced GnRH-induced GTH-II response in static incubation studies (Jobin *et al*, 1996a). However, similar treatments in perifusion experiments did not result in any changes in GnRH-induced GTH-II release (Jobin *et al*, 1996a). These results suggest the participation of CaM in mediating long-term rather than acute GTH-II response to GnRH in goldfish pituitary cells.

I.3.6. *Phospholipase A_2 and arachidonic acid.*

Arachidonic acid (AA) can be generated from DAG through the action of DAG lipase or by the direct action of phospholipase A_2 (PLA_2) on membrane phospholipids (reviewed in Noar, 1990). Results with inhibitors of DAG lipase and PLA_2 enzymes indicate that in goldfish, mobilization of AA through the PLA_2 pathway, but not the DAG lipase pathway, is involved in sGnRH stimulation of GTH-II release (Chang *et al*, 1994). Experiments with enzyme inhibitors of AA metabolism further indicate that lipoxygenase metabolites of AA mediate sGnRH-induced GTH-II secretion from goldfish pituitary cells. However, results from similar studies with cGnRH-II indicate that AA does not participate in cGnRH-II-stimulated GTH-II release (Chang *et al*, 1991c, 1994, 1995). Thus, AA is an important component of the sGnRH, but not cGnRH-II, signal transduction pathway in goldfish gonadotropes.

I.3.7. *Cyclic adenosine monophosphate (cAMP).*

Past studies with goldfish pituitary cells showed that GnRH-induced GTH-II release does not involve a cAMP-dependent component, although elevation of cAMP levels can increase GTH-II release. In the presence of IBMX, a phosphodiesterase inhibitor, neither sGnRH nor cGnRH-II increased cAMP accumulation, indicating that cAMP production was not stimulated by either of the two native GnRH forms (Chang *et al*, 1992). In addition, a PKA inhibitor, H89, was unable to affect the sGnRH- and cGnRH-II-induced GTH-II release (Jobin *et al*, 1996b). Lending further support to the non-involvement of cAMP-mediated mechanisms in GnRH stimulation of GTH-II secretion, cAMP-induced GTH-II release was also additive to the GTH-II responses to sGnRH, cGnRH-II, and activators of known GnRH signal transduction pathways (PKC and AA; Jobin *et al*, 1996b).

Though cAMP is not a component of GnRH signal transduction mechanism, it may modulate GnRH-stimulated GTH-II secretion in goldfish via actions on VSCC. Application of 8-Br-cAMP was shown to increase the magnitude of Ca^{2+} current flowing through VSCC in goldfish gonadotropes (Van Goor, 1997). In the presence of either 8-Br-cAMP or the adenylate cyclase activator, forskolin, the GTH-II responses to sGnRH and cGnRH-II were also enhanced (Chang *et al*, 1992). Thus, cAMP is likely to play a modulatory role in enhancing GnRH-stimulated GTH-II secretion in goldfish.

I.4. Transduction mechanism mediating DA action on GTH-II release in goldfish pituitary cells.

DA is an important inhibitory regulator of basal and GnRH-stimulated GTH-II release in goldfish (discussed in section I.2.2.2). The mechanisms of action of DA involve modulation of GnRH receptor numbers in the pituitary, as well as interference at different sites along GnRH signal transduction pathways.

I.4.1. *GnRH receptors.*

In goldfish, pituitary GnRH receptor numbers are regulated by DA actions. Injections of domperidone, a DA-D_2 receptor antagonist, resulted in a dose- and time-related increase in the binding capacity, but not affinity, of both the high and low affinity GnRH binding sites. Conversely, application of apomorphine, a DA agonist, decreased GnRH binding capacity (de Leeuw *et al*, 1989). Similar results were observed in *in vitro* treatment with DA agonists and antagonists. Treatment of goldfish pituitary fragments *in vitro* with apomorphine decreased the capacity of both high and low affinity GnRH binding sites without alterations in the binding affinity. Co-

treatment with domperidone reversed this effect (de Leeuw *et al*, 1989). It is likely that DA causes a down-regulation of both the high and low affinity GnRH-binding sites, which would reduce the effectiveness of GnRH in stimulating GTH-II release.

I.4.2. *GnRH signal transduction pathway.*

DA also inhibits GnRH stimulation of GTH-II release by actions on GnRH signal transduction pathways. DA-D_2 receptor activation reduced the GTH-II response to Bay K 8644 but not the response to a Ca^{2+} ionophore, A23187. DA, apomorphine, and the D_2 agonist LY 171555 were also effective in reducing Ca^{2+} currents through VSCC in goldfish gonadotropes (Van Goor, 1997; Van Goor *et al*, 1995). The inhibitory actions of DA on Ca^{2+} currents and GTH-II release were blocked by the DA-D_2 receptor antagonist, spiperone (Van Goor, 1997; Van Goor *et al*, 1995). In addition, LY 171555, inhibits the cGnRH-II-induced increase in $[Ca^{2+}]_i$ (Chang *et al*, 1993). These results suggest that DA, through D_2 receptor, inhibits GnRH-stimulated GTH-II secretion by modulating VSCC function but not by action at sites distal to Ca^{2+} mobilization.

In addition to inhibition of Ca^{2+} entry, LY 171555 also reduced the TPA-elicited GTH-II secretion from goldfish pituitary cells *in vitro* (Chang *et al*, 1993). cAMP-stimulated GTH-II release is also affected by the addition of the DA agonist apomorphine (Chang *et al*, 1992). These results suggest that DA inhibition is exerted at sites along the PKC- and cAMP-dependent GTH-II release mechanism (Jobin and Chang, 1993). However, AA-induced GTH-II release is not affected by apomorphine treatment, indicating that DA inhibition of GTH-II release does not involve modulation of AA-dependent mechanisms (Chang *et al*, 1991c).

I.5. Hormonal changes during the reproductive cycle in goldfish.

In temperate climate, goldfish reproduces on a seasonal cycle, spawning occurring when environmental conditions ensure maximum reproductive success. Photoperiod and temperature affect gonadal growth and maturation (Hontela and Stacey, 1990). Long photoperiod and cold temperature stimulate gonadal growth while extended exposure to long photoperiod and warm temperature induce ovarian atresia. Thus, gonadal development/recrudescence usually occurs in late fall and winter. Spawning occurs in the spring and the gonads remain regressed during the summer and early fall. Changes in environmental cues during the year probably control hormonal secretion profiles leading to gonadal development, maturation, and spawning. Changes in hormonal profiles during the reproductive cycle will be briefly discussed below.

I.5.1. *Seasonal hormonal changes: long-term changes.*

I.5.1.1. Females.

In female goldfish, GTH-II secretion rate is usually low during early stages of gonadal recrudescence in the fall. Plasma and pituitary levels of GTH-II increase with decreasing temperature as winter approaches (Hontela and Stacey, 1990). As gonadal recrudescence progresses through early winter to spring, ovarian sizes in females also increase as indicated by elevations in the gonadosomatic index (GSI; Kagawa *et al*, 1983). The maximal GSI in female goldfish is usually observed around the time of ovulation. After ovulation and spawning, circulating GTH-II levels usually remain low. Goldfish raised in simulated natural conditions have seasonal reproductive changes corresponding to those seen in feral female goldfish (Munkitterick and

Leatherland, 1984). The changes in plasma GTH-II levels seen during progressive ovarian development reflects increases in the secretion rate of GTH-II from the goldfish pituitary (Cook and Peter, 1980).

Plasma sex steroid levels also change on a seasonal basis in response to GTH-II secretion. Plasma level of E_2 is low from December to February, but it rapidly increases in March and is maintained at high levels in April (Kagawa *et al*, 1983). T levels significantly increase in March and remain at high levels during ovulation in April (Kagawa *et al*, 1983). The concentration of T in plasma is higher than that for E_2 from January onwards till after the spawning season. Progestogens (progesterone, 17α-hydroxyprogesterone, 17, 20ß P) levels increase prior to ovulation and usually rapidly decrease just after ovulation (Kagawa *et al*, 1983). The increase in plasma E_2 levels in December to March is likely due to the production of E_2 by the vitellogenic follicles. The increase in plasma T levels as compared to E_2 levels in March and April reflects changes in steroidogenic pattern of the mature follicles. Mature follicles (tertiary yolk stage) have been shown to produce mainly T but not E_2 (Kagawa *et al*, 1984).

Seasonal changes in plasma GTH-II levels are partly a consequence of changes in the neuroendocrine control of GTH-II release. Hypothalamic and pituitary sGnRH contents are lower in sexually mature than in sexually regressed female goldfish, possibly reflecting an increase in GnRH release in sexually mature goldfish (Yu *et al*, 1987; Rosenblum *et al*, 1994). The pituitary GnRH binding capacity and GTH-II release responsiveness to GnRH analogs are also highest at the periods of late gonadal recrudescence in goldfish during March and April (Habibi *et al*, 1989a). These changes in GnRH secretion, GnRH receptor numbers, and responsiveness to GnRH probably mediate the seasonal changes in GTH-II release. Pituitary DA turnover also increases as fish undergo gonadal recrudescence (Sloley *et al*, 1991, 1992). This increase in inhibitory DA tone keeps the circulating plasma GTH-II level from becoming too high (Sokolowska *et al*, 1985). These changes in the hypothalamic

control of pituitary GTH-II release is likely a consequence of changes in the circulating level of gonadal steroids and their effects on the hypothalamus-pituitary axis.

Concentrations of brain and pituitary aromatase, 5α-reductase, and androgen receptors change during the seasonl reproductive cycle, suggesting that gonadal steroid action on the brain-hypothalamus-pituitary axis changes with gonadal maturation. Aromatase activity and androgen receptor levels generally increase with gonadal maturation, reaching the highest levels during spawning season (Pasmanik and Callard, 1988a, b). A transient elevation in aromatase activity is also seen in the pituitary of female goldfish at the beginning of sexual recrudescence (Pasmanik and Callard, 1988a). On the other hand, 5α-reductase levels are lowest during spawning, and highest when circulating levels of gonadal steroids are low in sexually regressed fish (Pasmanik and Callard, 1988a). Since non-aromatizable androgens also have low binding affinities to androgen receptors (Pasmanik and Callard, 1988b), these results indicate that aromatizable androgens, estrogens, and progestogens, rather than non-aromatizable androgens, play a role in the modulation of the hypothalamic-pituitary axis during gonadal recrudescence.

Based on the known changes in plasma gonadal steroid levels, the gonadal steroid effects on hypothalamic neuroendocrine regulators (section I.2.5.3) and GnRH-induced GTH-II release (to be discussed in section I.5.2), a model of gonadal influences on the changes in the neuroendocrine control of GTH-II secretion during gonadal recrudescence in goldfish can be postulated. In sexually regressed fish, decreased DA turnover in the POA in response to T may release the DA inhibition on GnRH neurons, signalling the onset of gonadal recrudescence. The continued increase in the production of E_2 and T during gonadal recrudescence promotes further activation of the GnRH neuronal system. This is achieved by actions at the level of the hypothalamus and higher brain centers via influence on GABA synthesis and NPY activity. Since the ratio of circulating T:E_2 is low, the relative abundance of E_2 may

promote NE turnover at the level of the POA, further enhancing GnRH neuronal activity. However, the relatively high E_2 level inhibits NPY action at the level of the pituitary reducing the effectiveness of the stimulatory influence of NPY. These events promote a moderate increase in GnRH stimulatory influence but not the direct stimulatory action of NPY on gonadotropes. At later stages of gonadal recrudescence (post-vitellogenic), gonadal production of T increases at the expense of E_2. This change in T:E_2 ratio removes the inhibitory influence of E_2 on NPY action at the level of the pituitary and thus increases the stimulatory action of GABA. This leads to further increases in the stimulatory neuroendocrine drive on GTH-II release in sexually mature goldfish. The change in steroidal milieu may also enhance the effectiveness of GnRH to induce GTH-II release. Although the increase in GABA synthesis throughout gonadal recrudescence has a tendency to decrease DA turnover, the combined action of E_2 and T tends to increase DA turnover and elevate the inhibitory DA tone on the system. Thus, a balance of changing stimulatory and inhibitory hypothalamic influences is achieved. This prevents excessive GTH-II secretion while allowing for a general increase in stimulatory influence during the gonadal recrudescence process. The plasma GTH-II levels in circulation throughout the seasonal cycle is a balance of stimulatory and inhibitory influences on the brain-pituitary system.

I.5.1.2. Males.

In male goldfish, testicular size increases gradually from October through April, and decreases after spawning (Kobayashi *et al,* 1986a). The plasma androgen levels slowly increase as the testicular development progresses towards the spawning season. Plasma GTH-II levels in males increase during the spawning season in April during which the levels of 11-KT and T are also maximal. 17, 20β P level in plasma remains low during gonadal recrudescence, but increases as spawning season

approaches. There is also a switch from the production of 11-oxygenated androgens to the production of 17, 20β P during the final stages of gonadal development (Abdullah and Kime, 1994). Seasonal changes in aromatase activities in brain, pituitary, and testes of male goldfish are also observed. A peak in aromatase activity is seen during April in the POA and testes, while the greatest aromatase activity is observed in March in the pituitary (Pasmanik and Callard, 1988a). On the other hand, the maximal 5α-reductase levels in the brain, pituitary, and testes occur during gonadal regression (Pasmanik and Callard, 1988a). This suggests that a predominance of aromatizable androgens occurs during testicular maturation while other nonaromatizable androgens might be more predominant during sexual regression. As in the females, changes in neuroendocrine control of GTH-II secretion and steroid feedback probably mediate seasonal differences in hormonal profiles in the male.

I.5.2. *Short-term changes around the spawning period.*

Besides seasonal changes, hormonal levels also change transiently during spawning in goldfish. A major event associated with ovulation in females and increased milt production in males during spawning is a rapid elevation in plasma GTH-II levels (Kobayashi *et al*, 1986b, c, d). Ovulation in sexually mature female goldfish held on a long photoperiod (14 or 16 hours of light) can be induced by an increase in temperature. This spontaneous ovulation is preceded by a preovulatory GTH-II surge (Stacey *et al*, 1979).

In ovulating goldfish, significant decreases in GnRH concentrations in the olfactory bulbs, telencephalon, hypothalamus, and pituitary are correlated with increases in serum GTH-II levels (Yu *et al*, 1987, 1991). These changes in GnRH levels return to normal after ovulation. This suggests that the GnRH neuronal system is activated during spontaneous ovulation, leading to increased GTH-II release. A

decrease in the inhibitory DA influence on GTH-II release has also been observed during the periovulatory period (Sloley *et al*, 1991; Sokolowska *et al*, 1985). Plasma sex steroid levels change during the periovulatory period. Plasma levels of E_2 rise gradually to a moderate level during the GTH-II surge (Kobayashi *et al*, 1986c, 1987). T levels increase rapidly prior to the peak of GTH-II surge, and fall rapidly at the time of ovulation (Kobayashi *et al*, 1986c, 1987). Changes in the plasma levels of 17, 20ßP are rapid, reaching a maximum in the first half of the GTH-II surge. The close correlation of plasma steroid levels with the GTH-II surge suggests the existence of a relationship between these two events. The ability of implantation of T, but not E_2, to induce a GTH-II surge in ovariectomized and sexually regressed female goldfish suggests that high plasma level of T is a physiological pre-requisite for the occurrence of the ovulatory GTH-II surge (Kobayashi *et al*, 1989). Taken together with the enhanced ability of post-vitellogenic follicles to produce T and the rapid increase in T levels prior to the ovulatory GTH-II surge, these evidence strongly indicate a role for the positive feedback action of T in regulating the GTH-II increase observed during ovulation.

In male goldfish, changes in plasma levels of gonadal steroids are also evident during spawning. T levels increase sharply, reaching a peak during the period of ejaculation. These events occur simultaneously with the GTH-II surge in spawning males (Kobayashi *et al*, 1986d). Plasma levels of 17, 20ß P increase at the same time as the GTH-II surge in males, whereas 11-KT levels remain low (Kobayashi *et al*, 1986d). Injections of human chorionic gonadotropin (hCG) have been used to mimic the GTH-II surge seen during spawning in male goldfish (Kobayashi *et al*, 1986c). Following hCG treatment, milt production increases. This is accompanied by increases in T and 17, 20ß P, but not 11-KT, levels. Since increasing levels of T and 11-KT are observed during early testicular development, this suggests that T and 11-KT are likely involved in spermatogenesis. On the other hand, 17, 20ßP levels increase during the

final stages of testicular development, suggesting the possible involvement of 17,20ßP in mediating the GTH-II surge which is necessary for spermiation (Kobayashi *et al*, 1986a, c, d).

The synchronous release of milt from males and oocytes from females is essential for successful fertilization. This is in part achieved by a dual, time-dependent hormone-pheromone system. Ovulating female goldfish releases two sex pheromones that affect male behaviour and physiology. 17, 20ßP not only mediates final oocyte maturation, but is also released by the female just prior to ovulation and acts as a preovulatory "priming pheromone" to increase male "readiness" (Sorenson and Stacey, 1991). 17, 20ßP pheromone increases GTH-II release and milt volume in male goldfish (Sorenson and Stacey, 1991). 17, 20ßP action on GTH-II release and milt volume is proposed to be mediated by the removal of DA inhibition and an increase in GnRH stimulation on pituitary gonadotropes (Zheng and Stacey, 1997). 17, 20ßP also increases male goldfish fertility by increasing sperm quality (Zheng, 1996). During the onset of spawning activity, female goldfish release prostaglandins $F_{2\alpha}$ (PGF). PGF acts as a "releaser pheromone" and increases sexual arousal in males (Sorensen *et al*, 1988). PGF and other spawning cues also stimulate GTH-II release through actions on GnRH secretion (Zheng and Stacey, 1997). Hence, both the ovulatory process in females and milt production in the males depend on the well-coordinated endocrine events mediated by gonadal steroids and sex pheromones.

I.6. Feedback effects of gonadal steroids in other teleosts.

Steroid feedback regulation of GTH-II release has been demonstrated in teleost species other than the goldfish. This evidence will be briefly described below.

I.6.1. *Rainbow trout.*

Among salmonids, much of the information on gonadal steroid feedback action is from studies on the rainbow trout.

I.6.1.1. Males.

A negative feedback effect of sex steroids on GTH-II secretion has been demonstrated by castration of mature male rainbow trout (Billard *et al*, 1977; Billard, 1978). Castration increases plasma GTH-II levels; this effect is most prominent during the spawning season. These castration-induced elevations in GTH-II levels are suppressed by intraperitoneal implantations of T, E_2 or 11-KT (Billard *et al*, 1977; Billard, 1978). These results suggest that negative feedback effects of gonadal steroids exist in male rainbow trout. The intensity of the negative feedback is most prominent during the spawning season.

Positive feedback effects of gonadal steroids have also been demonstrated. Normally in immature trout, very low amounts of GTH-II could be detected in the pituitary. Intraperitoneal implantations of T into sexually immature male rainbow trout increases pituitary GTH-II content and plasma GTH-II levels (Crim and Evans, 1979, 1983). These results suggest that T stimulates the early development of gonadotropes in immature trout. The application of the aromatase inhibitor ATD significantly reduces the pituitary GTH-II increase in response to T treatment (Crim *et al*, 1981). The ability of T to elevate pituitary GTH-II levels in immature trout is also specific to aromatizable androgens and estrogens; neither nonaromatizable androgens nor progestogens have any effects (Crim *et al*, 1981). Thus, only aromatizable androgens and estrogens play a role in the positive feedback effect of steroids on pituitary GTH-II content in immature rainbow trout. T treatment increases GTH-II content in the pituitaries grafted from untreated fish (Gielen and Goos, 1983), indicating that the positive effects of gonadal steroids can be exerted directly at the level

of the gonadotropes. In male trout, onset of spermatogenesis is also observed with the T treatments (Crim and Evans, 1983). Taken together, these observations imply that T exerts a positive feedback effect on GTH-II release which is involved in stimulating precocious sexual development in male rainbow trout.

Testicular steroids from rainbow trout can also modulate the GTH-II response to sGnRH. Both 11-KT and 17,20ßP increase the GTH-II release response to sGnRH in pituitary cells prepared from male rainbow trout at early stages of spermiation. 17,20ßP is also effective in increasing sGnRH action on GTH-II release from pituitary cells prepared from spermiating males. In contrast, 17,20ßP exerts an inhibitory effect on sGnRH-induced GTH-II release from pituitary cells obtained from pre-spermiating males (Weil and Marcuzzi, 1990b). Hence, 11-KT and 17,20ßP have very different effects on pituitary response to sGnRH, depending on the stages of spermatogenesis.

As described earlier in section I.2.1, variation in pituitary GTH-I and II mRNA levels exists throughout testicular development in male rainbow trout (Weil *et al*, 1995). The GTH-II responsiveness of the pituitary to sGnRH also differs in the different stages of testicular development (discussed in the paragraph above). It is possible that testicular steroids play a role in modulating gene expression of GTH-I and II and their responsiveness to sGnRH stimulation during testicular development. Based on the findings observed by Weil and her colleagues, a model can be postulated. At the early stages of spermatogenesis, both 11-KT and 17,20ßP enhance basal GTH-I and II mRNA levels, particularly GTH-I levels. The GTH-II response to sGnRH is also enhanced by these two testicular steroids at this stage. At prespermiation, GTH-II mRNA levels slowly increase. But sGnRH-stimulated GTH-II release from the pituitary is reduced by 17,20ßP. This prevents the circulating levels of GTH-II from becoming too high. At the time of spermiation, both plasma 11-KT and 17,20ß P levels increase. However, the two increases are out of phase, with the level of 11-KT peaking

while the level of 17,20ß P is beginning to increase. These increasing levels of 11-KT and 17,20ß P also induce the synthesis of GTH-II mRNA which is observed to be at its highest level during spermiation. In addition, the sGnRH-stimulated GTH-II release is enhanced by the rising level of 17,20ßP. The increase in GTH-II release at this time of testicular development is possibly under the influence of 17,20ß P. Thus, in male rainbow trout, the changes in pituitary GTH-II release during testicular development is likely the consequence of changes in circulating testicular steroids .

I.6.1.2. Females.

Evidence for the presence of negative feedback effects of gonadal steroids in female trout also exists in the literature. Ovariectomy in female trout elevates plasma GTH-II levels. Replacement with E_2 reverses the ovariectomy-induced increase in GTH-II levels in fish at stages of oocyte maturation, but not in those at the end of vitellogenesis (Bommelaer *et al*, 1981). This suggests that a negative feedback effect of E_2 exists, at least at later stages of ovarian maturation in female rainbow trout. Since the plasma level of E_2 falls before the preovulatory GTH-II surge (Scott *et al*, 1983), it is likely that a reduction in the intensity of sex-steroid feedback inhibition, in part, allows the hypothalamic-mediated GTH-II surge to occur.

Gonadal steroids also modulate GTH-II synthesis in female rainbow trout. Administration of T in juvenile animals (Crim and Evans, 1979), or treatment of pituitary cells obtained from juvenile animals with T, E_2, and other aromatizable androgens increased pituitary GTH-II content (Crim *et al,* 1981). E_2 similarly increased pituitary cellular GTH-II content when applied to sterile, triploid adults (Breton and Sambroni, 1996), or animals at early stages of vitellogenesis (Weil and Marcuzzi, 1990a). These positive influences of T and E_2 are, at least in part, the results of direct stimulatory influences on GTH-II β-subunit mRNA synthesis in gonadotropes.

Estrogen receptors (ER) are present and the promoter region of the GTH-II β-subunit gene contains several estrogen responsive elements in trout gonadotropes (Xiong *et al*, 1994; Trinh *et al*, 1986). Whether other gonadal steroids also enhances GTH-II synthesis is unknown. Aromatization to estrogen may not be essential for androgens to exert this positive action in all cases. ATD treatment does not block the action of T in triploid adults (Breton and Sambroni, 1996). The stimulatory action of T and E_2 on GTH contents is specific for GTH-II since they do not affect GTH-I β-subunit mRNA levels (Xiong *et al*, 1994).

In addition to their effects on GTH-II synthesis, gonadal steroids also affect GnRH production and pituitary responsiveness to GnRH. T and E_2 treatment increase sGnRH content in the POA and pituitary of triploid adult females (Breton and Sambroni, 1996). 17,20ß P and E_2 increase the GTH-II release responsiveness to sGnRH in pituitary cells obtained from females at early stages of vitellogenesis and preovulatory females, but not from cells prepared from females at the time of ovulation (Weil and Marcuzzzi, 1990a). These steroid effects on GTH-II release responsiveness to GnRH and on brain GnRH content may explain the ability of T and E_2 implantation to elevate serum GTH-II levels in triploid adult females (Breton and Sambroni, 1996).

Taking into account the known distribution of ER in the rainbow trout brain and pituitary (Salbert *et al*, 1991; Anglade *et al*, 1994; Linard *et al*, 1995), changes in GTH-I and GTH-II levels during ovarian maturation (Weil *et al*, 1995), as well as the effects of gonadal steroid described above, possible roles of ovarian steroids in mediating the sexual maturation of female rainbow trout can be proposed. In juvenile trout and adult females at early stages of vitellogenesis, plasma GTH-I level is high. This is reflected in the dominance of GTH-I β-subunit mRNA in the pituitary. GTH-I stimulates early gonadal steroidogenesis resulting in an increased production of E_2. The increasing levels of E_2, together with other gonadal steroids, increase sGnRH synthesis in the brain and GnRH neuronal activity through activation of ER located at

the level of the ventral telencephalon, POA, and the medial basal hypothalamus. These effects cause an increase in plasma GTH-II level and a switch from a GTH-I to a GTH-II dominant stage. This promotes further ovarian development beyond the vitellogenic stage. Gonadal steroid effects at the hypothalamic level probably also alter the activity of DA neurons, which are known to contain ER (Linard *et al*, 1995). DA inhibition is important to prevent the excessive secretion of GTH-II. At the post-vitellogenic and the preovulatory period, plasma level of 17,20ß P increases and E_2 decreases due to changes in ovarian steroidogenesis under the influence of GTH-II. Increases in circulating 17,20ß P further enhance the pituitary responsiveness to GnRH. This, together with the removal of E_2 negative feedback action, could trigger the ovulatory GTH-II surge which is necessary for the induction of final oocyte maturation. Thus, similar to the situation in the goldfish, gonadal development in female rainbow trout is under the control of the positive and negative feedback actions of gonadal steroids at different levels of the HPG.

I.6.2. *Coho salmon.*

Evidence for a role of gonadal steroids feedback in the control of GTH-I and II synthesis and secretion is also available for other salmonids. Such findings in the coho salmon will be briefly described. Like the rainbow trout, plasma and pituitary levels of GTH-I increase at the early stages of oocyte growth and spermatogenesis. Plasma GTH-II levels are undetectable and pituitary GTH-II levels are low in fish at these maturational stages (Swanson, 1991). In male coho salmon at early stages of spermatogenesis, *in vivo* T or E_2 injections decrease GTH-I β gene transcript levels in the pituitary but do not affect plasma GTH-I levels (Swanson and Dickey, 1996). However, GTH-II β mRNA levels are increased by similar treatments with T or E_2 (Dickey and Swanson, 1995). In female fish at early stages of secondary oocyte

growth, *in vivo* T or E_2 injections have no effects on the GTH-I β-subunit mRNA levels, but increase GTH-II β-subunit mRNA levels in the pituitary. At later stages of secondary oocyte growth, treatment with T or E_2 decrease the plasma levels of GTH-I without altering GTH-I β gene transcript levels, and increase GTH-II β gene transcript levels without enhancing plasma GTH-II levels (Swanson and Dickey, 1996). Thus, GTH-I and II synthesis and secretion are differentially regulated by gonadal steroids at different stages of sexual development. GTH-I synthesis and secretion predominates during early gonadal development. With the increased maturation of the gonads, gonadal steroid action favours GTH-II synthesis and secretion.

I.6.3. *African catfish.*

Results from castration experiments also demonstrate the existence of negative feedback actions of gonadal steroids in the African catfish (*Clarias gariepinus*). Castration of adult male African catfish results in increased GTH-II levels in the plasma, decreased pituitary GTH-II contents, degranulation of gonadotropes, and increased pituitary GnRH receptor contents with no change in receptor affinity (de Leeuw *et al,* 1987; Habibi *et al,* 1989a). Replacement with T counteracts these changes (Habibi *et al,* 1989a). A more recent study in male catfish indicates that 11-KT also exerts a negative feedback effect on sGnRH analog-stimulated GTH secretion and these effects appear to be based on direct actions on the pituitary (Schulz *et al,* 1993). Taken together, these results suggest that gonadal androgens exert negative feedback control over the sensitivity of gonadotrope to GnRH stimulation in adult African catfish.

Besides negative feedback action, gonadal steroids also exert positive control over GTH-II synthesis directly at the level of the pituitary cells in this species. Incubation of pituitary cells for 48 h with E_2, T, 11-KT and the 11-oxygenated metabolite, 11β hydroxyandrostenedione (11OHA), elevated both GTH α- and GTH-II

β-subunit steady state levels. E_2, T, and 11-KT also increased *de novo* GTH-II synthesis, but 11OHA had the reverse effect on *de novo* GTH-II synthesis (Rebers *et al*, 1997). Since the action of T is abolished by ATD, these results suggest that estrogens, aromatizable androgens, and non-aromatizable androgens stimulate GTH-II synthesis. An additional site of steroid control may also be exerted at the level of hormone degradation or mRNA activity.

I.6.4. *European eel.*

As in other teleosts, gonadal steroids likely play a role in the maturation of the HPG axis in the European eel. In immature European freshwater eel, intraperitoneal injections of E_2 increase the low pituitary GTH-II contents (Dufour *et al*, 1983). T and E_2-treatments in males, and E_2-treatments in females, similarly cause an increase in pituitary GTH-II levels (Dufour *et al*, 1983). A more recent study using female eels shows that gonadal steroids differentially regulate the levels of the two endogenous GnRHs, LHRH and cGnRH-II. E_2 positively modulates pituitary and brain LHRH contents; in contrast, T negatively affects cGnRH-II levels in the brain (Montero *et al*, 1995). The ability of GnRH peptides (LHRH, cGnRH-II or LHRH-A) to stimulate GTH-II release *in vitro* was only observed from pituitary cells prepared from female eel pretreated with E_2 *in vivo* and not from cells prepared from control animals (Montero *et al*, 1996). Since LHRH neurons from the POA are known to project to the pituitary, E_2 may play an important role in enhancing GTH-II synthesis in the pituitary, GnRH content in the hypophysiotropic area, and pituitary GTH-II release sensitivity to GnRH stimulation during reproductive maturation.

I.6.5. *Other teleosts.*

Examples of gonadal steroid feedback action are also available for other cypriniform and perciform fishes. The presence of a positive feedback effect by gonadal steroids is demonstrated in common carp (*Cyprinus carpio*) and Chinese loach (*Paramisgurnus dabryanus*). Implantation of T, but not E_2, potentiates LHRH-A stimulated GTH-II release in sexually recrudescent and mature fish (Trudeau *et al*, 1991a). In addition, long-term T treatment stimulates the expression of the gene encoding for GTH-II β-subunit gene expression, elevates pituitary GTH-II content, and increases both basal GTH-II secretion and GnRH-stimulated GTH-II release in black carp, *Mylopharyngodon piceus* (Gur *et al*, 1995; Yaron *et al*, 1995). The positive feedback effects of T in the black carp involve direct actions at the pituitary level to modulate GTH-II synthesis and secretion. In immature tilapia, T selectively stimulates GTH-I β mRNA levels (Rosenfeld *et al*, 1996). In mature tilapia, only low doses of T increase GTH-I β mRNA levels whereas GTH-II β mRNA levels were increased by T in a dose-dependent manner (Melamed *et al*, 1996).

Thus, both negative and positive feedback effects of gonadal steroids are present in various species of fish.

I.7. Feedback effects of gonadal steroids in mammals.

As indicated in section I.1, gonadal steroids exert both positive and negative influence on gonadotropin release in mammals. These effects can be mediated through alteration of LHRH release or can be exerted at the level of the pituitary via mechanisms independent of LHRH secretion. Some examples of these latter effects will be reviewed briefly in the sections below.

I.7.1. *LHRH secretion-dependent effects.*

In mammals, LHRH is the primary stimulatory hypothalamic neuroendocrine factor mediating cyclic GTH secretion. LHRH stimulates both the synthesis and release of LH and FSH through receptor mediated action. The secretion of LHRH is pulsatile in nature. In rats, changes in the frequency and magnitude of LHRH pulsatility in part determine the relative magnitude of the LH and FSH responses. In general, shorter GnRH pulse intervals favour LH production and longer intervals favour FSH production. Moreover, the LH, but not the FSH, synthesis response is also restricted to a narrow range of LHRH pulse amplitudes (Marshall *et al*, 1991).

In the laboratory rat, the ovulatory LH surge is modulated by E_2 positive feedback. The ovulatory LH increase is associated with increases in LH release pulse frequency, and LHRH secretion. This is also preceded by an increase in pituitary LHRH receptor number (Katt *et al*, 1985). The frequency of the episodic LH release has been positively correlated with the pattern of pulsatile LHRH release in other mammals, such as in the ovine system (Crowder and Nett, 1984). It is believed that the ovulatory LH surge in rats is also preceded by an increase in LHRH release pulse frequency. Recent results from steroid replacement studies in ovariectomized rats indicate that E_2 prevented the decrease in LHRH receptor mRNA content and receptor numbers following ovariectomy and that LHRH receptor upregulation mediates the E_2-induced LH surge (Bauer-Dantoin *et al*, 1995; Yasin *et al*, 1995). It is likely that the positive regulation of LHRH receptor number and receptor mRNA content by E_2 is, at least in part, mediated by its influence on pulsatile LHRH secretion. Pulsatile LHRH secretion is also required for the homologous regulation of LHRH receptor numbers by LHRH (Loumaye and Catt, 1982; Katt *et al*, 1985).

I.7.2. *Effects at the pituitary level.*

Gonadal steroids may also act directly at the level of the pituitary to modulate GTH release and synthesis, as well as gonadotrope responsiveness to LHRH. For example, E_2 not only stimulates LHRH receptor synthesis via hypothalamic action on LHRH secretion, it also enhances the effectiveness of exogenous LHRH in elevating LHRH receptor mRNA levels in the rat (Yasin *et al*, 1995). E_2 treatment *in vitro* also augments the LHRH-induced LH and FSH secretion responses in cultured rat pituitary cells (Kamel *et al*, 1987; Liu and Jackson, 1988). On the other hand, T treatment *in vitro* decreases LHRH-induced LH secretion (Kamel *et al*, 1987), but enhances FSH secretion and FSHβ subunit mRNA synthesis (Gharib *et al*, 1990; Kamel and Krey, 1991).

Some of the direct effects of gonadal steroids on pituitary GTH synthesis and release responsiveness involve actions on the LHRH intracellular signal transduction mechanisms. Several signal transduction pathways have been proposed to mediate LHRH action on mammalian gonadotropes, including PLC activation, mobilization of Ca^{2+} from intracellular and extracellular sources, AA mobilization and metabolism, PKC activation, and cAMP (Stojilkovic *et al*, 1994). In rat pituitary cells pretreated with E_2, the LH and FSH release responses to LHRH, Ca^{2+} ionophore, cAMP, PLC, and activators of PKC are enhanced (Liu and Jackson, 1988; Audy *et al*, 1990). *In vitro* E_2 treatment also augments LH glycosylation induced by Ca^{2+} ionophore, PLC, and activators of PKC (Liu and Jackson, 1990). However, E_2 does not affect AA-stimulated LH and FSH secretion (Liu and Jackson, 1988). Therefore, E_2 potentiates the effectiveness of selected LHRH second messenger systems to increase the hormone synthesis and secretion responses to LHRH. In rat pituitary cells, E_2 treatment has subsequently been shown to elevate PKC activity by increasing PKC synthesis (Drouva *et al*, 1990; Thomson *et al*, 1993), and to increase LHRH-stimulated InsP production (Ortmann *et al*, 1995). In addition to E_2, T and P_4 have also been shown to modulate GnRH-stimulated Ca^{2+} signalling (Ortmann *et al*, 1992, 1995;

Tobin and Canny, 1996). In ovine pituitary cells, E_2 alters the pattern of GnRH-induced increases in $[Ca^{2+}]_i$ in a fashion consistent with increased Ca^{2+} influx (Heyward and Clarke, 1995).

I.8. Research objectives: direct effects of T on GTH-II release in goldfish.

I.8.1. *Brief recapitulation of literature and justification.*

As shown in previous sections, gonadal steroids are important to the reproductive success in teleosts. The HPG axis is tightly regulated by the feedback actions of gonadal steroids throughout gonadal development and maturation. Negative feedback actions are more involved with the regulation of basal GTH-II release, whereas positive feedback is crucial for the maintenance of the elevated GTH-II levels during the periovulatory period and for priming the system for the ovulatory GTH-II surge. Understanding the mechanisms underlying these positive actions of gonadal steroids is an important step in understanding the neuroendocrine regulation of reproduction in teleosts.

In the goldfish, four lines of evidence indicate that T plays an important role in the positive feedback action on GnRH-stimulated GTH-II release, especially during later stages of gonadal maturation and in the preovulatory period. First, although the plasma level of E_2 is high during vitellogenesis, the mature follicles produce mainly T but not E_2 (Kagawa *et al*, 1983). Second, the concentration of T in serum is higher than that of E_2 during the later stages of gonadal recrudescence, from January onwards till after the spawning season (Kagawa *et al*, 1983). Third, serum T concentration rapidly increases just prior to the preovulatory GTH-II surge in females; the level of E_2 remains moderate throughout the GTH-II surge (Kagawa *et al*, 1983). Likewise, T

levels also peak during ejaculation in conjunction with the increase in GTH-II levels during spawning in males (Kobayashi *et al*, 1986d). Fourth, implants of T, possibly not E_2, were shown to induce a GTH-II surge in ovariectomized goldfish (Kobayashi *et al*, 1989). In addition, the positive feedback effect of T may be responsible for the activation of GnRH neuronal system during initiation of gonadal recrudescence (discussed in section I.5.1.1). Thus, T is likely a major factor exerting a positive feedback influence on the HPG during important stages in gonadal maturation and reproduction in the goldfish.

Previous studies have shown that part of the feedback action of T is exerted directly at the level of the pituitary by enhancing GnRH-stimulated GTH-II release from goldfish pituitary fragments (Trudeau *et al*, 1993a). However, functional nerve terminals remain in these fragments. As modulation of GnRH release from nerve terminals remaining in pituitary fragments by neurotransmitters and depolarization has been demonstrated (Yu *et al*, 1991), it is unclear whether the *in vitro* action of T is mediated indirectly through actions on the release of neuroendocrine factors present in the nerve terminals or is exerted directly at the pituitary cell level. Dispersed goldfish pituitary cells, which have been shown to be devoid of nerve terminals (Chang *et al*, 1990a), are therefore more suitable to study the direct effects of gonadal steroids on GnRH-stimulated GTH-II release. In such preliminary experiments, Lo *et al* (1995) demonstrated that prolonged T treatments of dispersed pituitary cells prepared from a mixture of male and female goldfish in early fall potentiated GTH-II responses to pulse applications of sGnRH or cGnRH-II in cell column perifusion experiments. Pretreatment with 0.1 μM T for 16 h enhanced the GTH-II response to 15-min pulses of 100 nM sGnRH or cGnRH-II regardless of the presence or absence of T during perifusion testing. In contrast, acute T treatments (i.e., T applied 15 min before, during, and for 15 min after the GnRH pulse) did not affect GnRH-induced GTH-II response. Basal GTH-II release or cellular GTH-II contents were not affected by T-

pretreatment. Thus, T is able to act directly at the pituitary cell level to potentiate GnRH-induced GTH-II response in the goldfish. However, whether this direct positive influence of T on pituitary cell responsiveness to GnRH can be observed at all times of the seasonal reproductive cycle is not known. In addition, little information is available concerning the mechanisms by which T exerts its potentiating effect on GnRH-induced GTH-II release. Such knowledge is necessary in order to evaluate the potential importance of the direct T positive feedback action at different reproductive stages, as well as to understand how the effects of T are manifested at the level of the pituitary cells. To understand these aspects, the present thesis research was conducted.

I.8.2. *Specific objectives and approaches.*

The two main objectives of this thesis are: 1) to examine if the ability of T to potentiate GnRH-stimulated GTH-II release by direct action on goldfish pituitary cells varies during the gonadal reproductive cycle, and 2) to elucidate the mechanisms by which these effects of T are achieved. Primary cultures of dispersed goldfish pituitary cells were used. Acute and long-term GTH-II release responses to agonists were examined using cell column perifusion studies and static incubation experiments, respectively. A protocol of T pretreatment for at least 24 h was chosen since acute exposures to T were shown to be ineffective in altering GTH-II release responses in preliminary studies (Lo *et al*, 1995). To achieve the two objectives, seven groups of experiments were performed.

First, the effects of T pretreatment on maximal sGnRH- and cGnRH-II-stimulated GTH-II release were examined in perifusion studies using pituitary cells of female goldfish obtained at different stages of the seasonal gonadal recrudescence cycle. At all gonadal developmental stages tested, T potentiated the GTH-II response to both goldfish native GnRH forms.

Second, the influence of T on GTH-II release responsiveness to different concentrations of sGnRH and cGnRH-II was examined in perifusion studies. This evaluates if T selectively alters the sensitivity of gonadotropes to either of the two endogenous GnRH forms.

Third, the ability of T pretreatment to affect total radioimmunoassayable GTH-II contents prior to, and following either sGnRH or cGnRH-II challenge, were investigated in static incubation experiments. This estimates whether the total GTH-II pool size is altered by T. In addition, it examines if T affects the ability of the two GnRHs to increase the size of the GTH-II pool. Thus, these data provide an indirect measure of whether GTH-II synthetic capacity has been altered by T. Results from these experiments also established the static incubation system as an alternative to perifusion for subsequent experiments on the possible mechanisms of T action on GTH-II release.

Fourth, the possible influence of T on PKC-mediated GTH-II release, an important component in the GnRH action, was examined. Following T pretreatment, PKC-activator-induced GTH-II release and the ability of a PKC inhibitor to attentuate GnRH-induced GTH-II secretion were monitored.

Fifth, the effects of T on Ca^{2+} ionophore-induced GTH-II release was examined. This provides information on the influence of T on the total Ca^{2+}-sensitive releasable GTH-II pool and the effects of increases in $[Ca^{2+}]_i$ on GTH-II exocytosis.

Sixth, the influence of T on VSCC agonist-stimulated GTH-II release and the ability of a VSCC antagonist to attenuate the GTH-II response to GnRH were examined. Results from these experiments provide an indication on whether T affects VSCC function and VSCC-dependent GnRH action.

Seventh, the possible involvement of aromatization in mediating T action on GnRH-induced GTH-II release was evaluated. The ability of two

nonaromatizable androgens, DHT and 11-KT, to mimic T action and that of an aromatase inhibitor, ATD, to inhibit the potentiating effects of T were examined.

Results presented in this thesis provide important insight into the effects of T at the level of the pituitary cells in goldfish. T can potentiate GnRH-induced GTH-II release by action at the pituitary cell level at all times of the seasonal reproductive cycle. This effect of T therefore likely requires aromatization of T and may be the result of a sensitizing effect on PKC-dependent GnRH action.

II. *Materials and Methods*

II.1. Animals.

Common goldfish, *Carassius auratus*, (4-5 inches in length) were purchased from Grassyforks Fisheries Co. (Martinsville, IN) and Ozark Fisheries, Inc. (Stoutland, MO). Immediately upon arrival in the laboratory, they were held in flow-through aquaria and acclimated to 17°C under simulated natural photoperiod (photoperiod adjusted weekly according to the time of sunrise and sunset in Edmonton, Alberta). Fish were used within 1 month of arrival. Fish were fed daily with trout pellets. Reproductive conditions of female goldfish used in experiments examining the effects of sexual maturation on T actions were determined at the time of experiments by their gonadosomatic index (GSI) [(gonadal weight / body weight) x 100%]. Female goldfish having GSI values of < 2% are considered as gonadally regressed, 2-6% as gonadally recrudescent, and >6% as gonadally mature (Munkittrick and Leatherland, 1984). In other experiments, when both male and female goldfish were used, the gonadal conditions were roughly estimated by the time of the year (see section I.5 for a description of the seasonal reproductive cycle of goldfish).

II.2. Steroids and test substances.

Stock solutions of T, DHT (Sigma, St. Louis, MO), 11-KT, ATD, (Steraloids Inc., Wilton, NH), and Bay K 8644 (Calbiochem, San Diego, CA) were made in 100% ethanol. sGnRH, cGnRH-II (Peninsula Laboratories Inc., Belmont, CA) and H7 (1-5-isoquinolinesulfonyl-2-methylpiperazine dihydrochloride; Calbiochem) were dissolved in deionized distilled water. Tetradecanoyl phorbol-13-acetate (TPA) and ionomycin (Calbiochem) were dissolved in dimethylsulphoxide.

Aliquots of drug solutions were kept frozen at -20°C. All drugs were diluted to the required concentrations with appropriate testing media prior to use. Tissue culture media, antibiotics, and horse serum were purchased from GIBCO (Grand Island, NY).

II.3. Cell dispersion and static culture incubation.

Fish were anaesthetized by immersion in 0.05% tricaine methanesulfonate (Syndel, Vancouver, B.C.) prior to decapitation. Pituitary glands were removed and digested enzymatically with trypsin/DNAase according to procedures described by Chang *et al.* (1990a). The dispersed cells were plated in 24-well culture plates with plating medium (medium 199 with Earle's salts, 2.2 g/l sodium bicarbonate, 25 mM HEPES, 100,000 U/l penicillin, 100 mg/l streptomycin, pH 7.2) at a density of 0.25 million cells per well in 800 µl of medium. Cells were incubated at 28°C, and under 5% CO_2, and saturated humidity. Two h after plating, steroids (at 5-times the desired final concentration) were added in 200 µl of plating medium with 5% horse serum to the appropriate culture wells. Non-steroid-treated wells received 200 µl of plating medium with 5% horse serum alone. Unless otherwise stated, cells were cultured for an additional 24 h before experiments on GTH-II release began. Culture wells without the further addition of steroids had estimated T and E_2 levels of 0.10 and 0.23 ng/ml (n = 2; Appendix 1), respectively, during this pre-testing incubation period. Prior to experiments, plating medium was collected and replaced with testing medium (medium 199 containing Hank's salts, 25 mM HEPES, 0.1% bovine serum albumin, 2.2 g/l sodium bicarbonate, 100,000 U/l penicillin, and 100 mg/l streptomycin, pH 7.2, and supplemented with the appropriate amount of steroids). Inhibitors and secretogogues were then added (1 µl per ml to achieve final desired concentration). Inhibitors were usually added 10 min prior to application of secretagogues. Cells were then returned to the incubator for the duration of secretion testing (either 30 min or 2 h).

At the end of the testing period, 800 µl of the medium was collected from each well. Where measurements of the GTH-II content remaining in the cells were required, the remaining testing medium was discarded and the cells lysed by the addition of 1 ml H_2O followed by freezing and thawing. All treatments were tested in triplicate or quadruplicate in each experiment. All samples were stored at -20°C until their GTH-II contents were measured by radioimmunoassay (RIA; see section II.6 on RIA below). GTH-II release into the medium was expressed either as a percentage of the levels observed in unstimulated control wells (% control) or as ng/ml. In cases where basal hormonal release was affected by T pretreatment, the net response to the secretagogues was calculated (net response = response in the presence of secretagogues - response without the secretagogue). Experiments were repeated up to 5 times. Results from replicate experiments were pooled prior to data analyses.

II.4. Cell column perifusion.

Cell column perifusion studies were performed under conditions modified from Chang *et al* (1990c). Briefly, dispersed cells were allowed to adhere to preswollen cytodex-I beads (Sigma) in plating media in petri dishes at 28°C, and under 5% CO_2 and saturated humidity. Steroid treatments began either after 2 h of plating or the next morning. After an additional 24 h of incubation, the cell/bead mixture was transferred to perifusion columns and perifused with testing medium (medium 199 with Hank's salts, 2.2 g/l sodium bicarbonate, 25 mM HEPES, 100,000 U/l penicillin, 100 mg/l streptomycin, 0.1% bovine serum albumin, pH 7.2, and the appropriate concentrations of added T) at a flow rate of 15 ml/h for 3.5-4 h to establish basal GTH-II secretion. Experiments commenced with the collection of perifusate in 5-min fractions. Treatments were tested in duplicate for each experiment. Perifusates were kept frozen at -20°C until their GTH-II contents were measured by RIA. GTH-II

release was normalized as a percentage of the average GTH-II values observed in the first 6 fractions collected at the beginning of an experiment (% pretreatment). This form of data transformation accounts for differences in basal hormone release rates between columns due to variations in cell loading. In addition, it allows for pooling of data from replicate columns without distorting the hormone release profile (Wong *et al*, 1992). Net GTH-II responses to secretagogues were quantified as the "area under the curve" as described by Wong *et al* (1992). Fractions having GTH-II values greater than the mean plus 1 SEM of values obtained in the three fractions obtained prior to each application of secretagogue were included as part of the hormone response. Experiments were repeated up to 5 times. Results from replicate experiments were pooled prior to data analysis.

II.5. Experimental protocols.

II.5.1. *Effects of 1 to 100 nM T on 100 nM GnRH-stimulated GTH-II release from perifused pituitary cells prepared from female goldfish at different reproductive stages.*

Dispersed pituitary cells were prepared from female goldfish at different stages of gonadal maturation: sexually regressed (summer and early fall), undergoing sexual recrudescence (late fall and early winter), and sexually mature (=prespawning; early spring). Cells were incubated in four different doses of T (either 0, 1, 10 or 100 nM) for 24 h prior to the transfer of the cell-bead mixture to the perifusion columns. At 30 min after the commencement of the experiment, the first 30-min GnRH (100 nM sGnRH or 100 nM cGnRH-II) pulse was applied. The cells were perifused in testing medium containing T for an additional 2.5 h before a second 30-min GnRH (100 nM sGnRH or 100 nM cGnRH-II) pulse was applied. The cells were then perifused in T-

containing testing medium for another 35 min. Perifusates were collected as 5-min fractions. To evaluate if T exerts similar effects on the GTH-II responses in pituitary cells from both males and females, these experiments were also repeated using cells prepared from both sexes at the time of prespawning.

II.5.2. *Effects of T on the dose-dependent GTH-II release response to sGnRH or cGnRH-II.*

Pituitaries from both male and female sexually regressed goldfish were used in these experiments. Cells were incubated in either 0, 10 or 100 nM of T for 24 h prior to the transfer of the cell-bead mixture to perifusion columns. At 30 min after the commencement of fraction collection, 5-min pulses of GnRH were applied at 1-hour intervals. Five doses (0.01, 0.1, 1, 10, and 100 nM) of sGnRH and cGnRH-II were tested in either ascending or descending order of concentration. This protocol of GnRH application has previously been shown not to cause potentiation or desensitization of sequentially applied GnRH pulses (Chang *et al*, 1990c; Jobin *et al*, 1996a).

II.5.3. *Effects of T on pituitary cell GTH-II contents and responsiveness to GnRH in static culture.*

The effects of T-treatment on total cellular GTH-II content available before and following GnRH challenge were examined in static incubation studies. Cells prepared from male and female fish at the prespawning or postspawning stage were cultured with either 0 or 10 nM T for 28 h. Culture medium was collected from some of the wells for measurements of GTH-II released during the culture period and the cells lyzed with deionized distilled water to release the remaining cellular GTH-II contents. For the remaining wells, the culture medium was replaced with testing medium having

the same concentration of T as in the culture period (either 0 or 10 nM T). They were then challenged with either 100 nM sGnRH, 100 nM cGnRH-II or vehicle for an additional period of either 30 min or 2 h. Testing medium was then collected for measurements of GTH-II released and the cells lysed with distilled deionized water to release the cellular GTH-II content.

II.5.4. *Effects of T on the effectiveness of signal transduction components mediating GnRH action.*

II.5.4.1. The involvement of PKC.

A PKC activator (TPA) and a PKC inhibitor (H7) were used to study the effects of T on the PKC-induced GTH-II release. Pituitary cells were prepared from male and female goldfish during the spawning season, and incubated with either 0 or 10 nM T under conditions described for static culture. Before the experiment, the plating medium was replaced with testing medium. Cells were then incubated for a further 2 h with TPA (0.01 to 100 nM), 100 nM sGnRH or 100 nM cGnRH-II, and either in the presence or absence of 10 μM H7.

II.5.4.2. Effects of T on Ca^{2+}-dependent GTH-II release.

The influence of T on Ca^{2+}-dependent GTH-II release was examined using a Ca^{2+} ionophore, ionomycin. Pituitary cells were prepared from postspawning male and female goldfish, and incubated with either 0 or 10 nM T for 24 h in static culture. Prior to experiments, cells were washed twice with testing medium. Cells were then incubated for an additional 2 h with 0.01 to 100 μM ionomycin.

II.5.4.3. Effects of T on the GTH-II release response to Ca^{2+} entry through VSCC.

Pituitaries were obtained from both male and female goldfish undergoing sexual recrudescence and the cells dispersed as described for static incubation studies. The dispersed pituitary cells were treated with either 0 or 10 nM T for 24 h in static culture. Following replacement of culture medium with testing medium, cells were incubated for 2 h with doses of Bay K 8644 ranging from 0.001 to 10 μM .

II.5.4.4. Effects of T on extracellular Ca^{2+}-dependent GnRH action on GTH-II release.

Pituitaries were obtained from male and female goldfish undergoing sexual recrudescence and the cells were dispersed and cultured on cytodex beads as described for perifusion studies. Dispersed pituitary cells were incubated in either 0 or 10 nM T for 24 h prior to transfer of the cell-bead mixture to perifusion columns. Thirty min after the commencement of experiments, a 5-min pulse of either 100 nM sGnRH or 100 nM cGnRH-II was applied. One h after the GnRH pulse, the cells were perifused with media containing 1 μM (for sGnRH experiments) or 10 μM (for cGnRH-II experiments) nifedipine. Both 1 and 10 μM doses of nifedipine have previously been shown to be effective in altering GnRH-stimulated GTH-II release in goldfish (Jobin and Chang, 1992b; Jobin *et al*, 1996a). Forty-five min after the commencement of nifedipine treatment, a second 5-min GnRH pulse was applied in the presence of nifedipine (some columns did not receive the second GnRH pulse). Cells continued to receive nifedipine-containing media for another 20 min after GnRH pulse application, making the entire duration of nifedipine treatment 70 min. The cells were

then perifused with T-containing media for 2 h before a third 5-min GnRH pulse (washout) was applied.

II.5.5. *Involvement of aromatization in the actions of T.*

The possible involvement of aromatization in the direct actions of T on pituitary GTH release was first examined using nonaromatizable androgens, 11-KT and DHT, in perifusion studies. Dispersed pituitary cells obtained from sexually regressed male and female goldfish were incubated in either 10 nM T, 10 nM 11-KT, or 10 nM DHT for 24 h. Control columns received no additional steroids. Cells were then loaded into perifusion columns. GTH-II release stimulated by pulse-application of GnRH was then examined. Two 0.5-h 100 nM sGnRH pulses were applied at an interval of 2.5 h.

Next, the effects of an aromatase inhibitor, ATD, on GTH-II release and cellular GTH-II contents were studied in static incubation. Dispersed pituitary cells were incubated in 0 or 10 nM T, either in the absence or presence of 300 μM ATD, for 24 h. Following replacement of culture medium by testing medium, 100 nM sGnRH or 100 nM cGnRH-II was added, and the cells incubated for an additional 2 h. Media were removed for measurements of GTH-II release and cells were lyzed with distilled deionized water to release the GTH-II contents.

II.6. RIA.

GTH-II contents in samples were determined using a double-antibody RIA for carp GTH-II which has been validated for measurements of goldfish GTH-II (Peter *et al*, 1984; Van Der Kraak *et al*, 1992). All samples were processed in duplicates. For measurement of pituitary GTH-II content, pituitaries were homogenized in barbital buffer (24.25 mM sodium barbital, 23.88 mM sodium acetate, 0.24 mM

thimersol, pH adjusted to 8.6 with HCl) and serial dilutions were made prior to the determination of GTH-II contents by RIA. Interassay variability of the assay, determined as the coefficient of variation of the concentration of GTH-II giving a 50% displacement in all standard curves, is 2.5% (n = 39). Intraassay variability of the assay, determined as the coefficient of variation of three separate determination of a 5 ng/ml sample within each assay run, is 8% (n = 39).

II.7. Statistical analyses.

Multiple comparisons of GTH-II contents in ng/ml were performed using ANOVA followed by Fisher's PLSD test (StatView SE+Graphicis, Berkeley, CA). Multiple comparisons of GTH-II release in % pretreatment or % control were performed using Kruskal Wallis Test followed by Dunn's test (Instats, San Diego, CA). Wilcoxon signed-rank tests were used to evaluate paired data and Mann-Whitney U tests were used for comparisons between two unpaired groups (StatView SE+Graphicis, Berkeley, CA). Differences were considered significant if $p<0.05$. Dose-response data were analyzed using the Allfit computer program (De Lean *et al*, 1978) to estimate the half-maximal effective dose (ED_{50}; potency) and the maximal response (efficacy); values having overlapping 95% confidence intervals are considered as not significantly different from one another.

III. *Results*

III.1. Effects of T on GnRH-stimulated GTH-II release throughout the seasonal reproductive cycle.

The possible existence of a potentiating effect of T on GnRH-stimulated GTH-II release throughout the reproductive cycle was initially examined in perifusion studies using dispersed goldfish pituitary cells obtained from female goldfish at different stages of gonadal maturation. The effects of preexposure to 1, 10, and 100 nM T were examined. These three doses of T closely approximate the range of circulating T levels found in female goldfish. In sexually regressed and mature female goldfish, the concentration of T in circulation has been reported to be ≈0.5 ng/ml (≈2 nM) and ≈28 ng/ml (≈100 nM), respectively (Huggard *et al*, 1996). In these experiments, two 0.5-h GnRH pulses were applied to investigate if the influence of T pretreatment on the GTH-II responses to GnRH would be similar for repeated GnRH challenges.

III.1.1. *Effects of T on 100 nM sGnRH-stimulated GTH-II release from pituitary cells prepared from female goldfish.*

Pretreatment with T did not alter basal GTH-II release from cells prepared from either sexually regressed, recrudescent, or mature goldfish (Figs. 1-6). In cells prepared from sexually regressed goldfish, preexposure to 100 nM T significantly increased GTH-II release in response to the first sGnRH pulse compared to controls (Fig. 1B). In control cells not pretreated with additional T (T = 0 nM group), the GTH-II responses to the first and second sGnRH pulses were similar, indicating that the GTH-II response in the control cells was not desensitized by prior

0.5-h sGnRH exposure ($p>0.05$; n = 4-6; Wilcoxon signed-rank test). However, none of the T treatments significantly increased the second sGnRH-stimulated GTH-II response compared to the corresponding control response and all responses to the second sGnRH challenge were smaller than their corresponding first responses. These results indicate that pretreatment with a high dose of T potentiated GTH-II release in response to an initial exposure to sGnRH in pituitary cells from sexually regressed female goldfish. However, the ability of T pretreatment to potentiate the GTH-II response to a second exposure to sGnRH was attenuated.

With pituitary cells from sexually recrudescent female goldfish, pretreatment with either 1, 10, or 100 nM T significantly increased the GTH-II response to the first pulse of sGnRH over that of controls (Fig. 2B). However, only 100 nM T significantly potentiated the GTH-II release in response to the second sGnRH application (Fig. 2B). The GTH-II responses to the first and second sGnRH pulse were similar in control cells indicating that desensitization of the GTH-II response did not occur in these untreated cells ($p>0.05$; n = 6-8; Wilcoxon signed-rank test). In contrast, in T-treated cells, the GTH-II responses to the second sGnRH pulse were significantly lower than those observed in the first pulse ($p<0.05$; n = 6-8; Wilcoxon signed-rank test). T-pretreatment resulted in a significant increase in the GTH-II response to an initial stimulation by sGnRH in pituitary cells from female goldfish undergoing sexual recrudescence. The potentiation action of T on the GTH-II response was diminished during a subsequent sGnRH challenge.

In pituitary cells obtained from prespawning female goldfish, both 10 and 100 nM T treatments significantly potentiated GTH-II release in response to the first sGnRH pulse (Fig. 3B). In control cells, the GTH-II response to the second sGnRH challenge was not significantly different from that to the first sGnRH pulse ($p>0.05$; n = 6-8; Wilcoxon signed-rank test). On the other hand, all three doses of T did not significantly potentiate the GTH-II responses to the second sGnRH pulse (Fig.

3B). The sGnRH-induced GTH-II responses in the second pulse were also significantly lowered in cells treated with 1 and 10 nM T ($p<0.05$; n = 6-8; Wilcoxon signed-rank test). T pretreatment enhanced the GTH-II responses to an initial sGnRH challenge in pituitary cells from sexually mature female goldfish, but an ability of T to significantly potentiate the GTH-II response was not observed upon a second stimulation by sGnRH.

III.1.2. *Effects of T on 100 nM cGnRH-II-stimulated GTH-II release from pituitary cells prepared from female goldfish.*

The effects of T on the GTH-II response to cGnRH-II were also investigated in pituitary cells obtained from sexually regressed, recrudescent, and mature female goldfish. In dispersed pituitary cells prepared from goldfish at all three gonadal maturational stages, 10 and 100 nM doses of T potentiated cGnRH-II-stimulated GTH-II release during the first GnRH pulse application (Figs. 4-6). 1 nM T was also effective in potentiating the GTH-II response to the first cGnRH-II pulse in experiments with sexually regressed fish (Fig. 4). In the second cGnRH-II pulse, all three doses of T did not enhance GTH-II responses in cells prepared from sexually regressed female goldfish (Fig. 4B). In cells prepared from sexually recrudescencing females, only 100 nM T significantly increased the GTH-II response to the second cGnRH-II pulse (Fig. 5B); both 10 and 100 nM T were effective in this regard in cells from matured females (Fig. 6B). However, compared to the GTH-II responses to the first cGnRH-II pulse, the GTH-II responses to the second cGnRH-II pulses were smaller in T-treated cells from sexually regressed, recrudescing and prespawning females (Figs. 4B, 5B and 6B; $p<0.05$; n = 6; Wilcoxon signed-rank test). In contrast, the GTH-II responses from control cells were similar between the two cGnRH-II pulses in all three gonadal maturational stages ($p>0.05$; n = 6; Wilcoxon signed-rank

test). These results indicate that T was effective in potentiating the GTH-II response to the first cGnRH-II pulses in pituitary cells from sexually regressed, recrudescent, and mature female goldfish. In addition, T also enhanced the GTH-II responses to a second cGnRH-II application in cells prepared from recrudescent and mature female goldfish. However, the degree of enhancement was diminished following a previous 0.5-h challenge with cGnRH-II.

III.1.3. *Effects of T on 100 nM sGnRH- and 100 nM cGnRH-II-stimulated GTH-II release from pituitary cells prepared from prespawning male and female goldfish.*

The effects of T on sGnRH- and cGnRH-II-stimulated GTH-II release were also examined at prespawning stages using pituitary cells prepared from both male and female goldfish. Basal GTH-II release was not affected by T pretreatments. Pre-exposure to 10 and 100 nM T significantly potentiated GTH-II response to the first pulse application of 100 nM sGnRH (Fig. 7) and 100 nM cGnRH-II (Fig. 8). These data indicate that T was effective in increasing GTH-II responses to both native GnRHs in cells prepared from prespawning males and females as well as with cells prepared from prespawning female goldfish alone (Figs. 3 and 6).

Prior exposure to sGnRH or cGnRH-II did not affect the magnitude of the GTH-II response to a subsequent challenge with the same GnRH peptide in control cells prepared from both prespawning males and females, but decreased the second response to T-pretreated groups (Figs. 7 and 8; $p>0.05$; $n = 4$; Wilcoxon signed-rank test). In the second sGnRH pulse, 1 and 100 nM T elevated the GTH-II response compared to controls (Fig. 7B). In the case of the cGnRH-II-induced release, 100 nM T was also effective in enhancing the response to the second GnRH pulse (Fig. 8B).

From these experiments, it was demonstrated that, in general, the ability of T-pretreatment to enhance the GTH-II responses to the two native GnRHs could be observed with either pituitary cells obtained from female fish alone or cells prepared from both male and female goldfish. In addition, the magnitude of the enhanced GnRH-stimulated GTH-II responses following pretreatment with the different doses of T were comparable between cells prepared from prespawning female goldfish alone and those prepared from both male and female fish at a similar sexual maturational stage.

III.1.4. Effects of T on the phasic GTH-II response characteristics in goldfish pituitary cells.

In goldfish pituitary cells, the GTH-II response to a prolonged (1 h) perifusion of sGnRH is "biphasic" (Wong *et al*, 1993). The "biphasic" GTH-II response consists of an initial rapid increase in GTH-II release rate lasting for approximately 15 min ("peak" phase), this is followed by a sustained elevation in release rate of lower magnitude for the rest of the duration of the release response ("plateau" phase). The two phases of the GTH-II response are mediated by dissimilar second messenger systems (reviewed in Chang and Jobin, 1994; Chang *et al*, 1996). GTH-II responses to the first 0.5-h application of sGnRH observed in the present study can similarly be separated into a "peak" (first 15 min) and a "plateau" phase (rest of the response). In this study, T consistently enhanced the GTH-II responses to the first 0.5 h GnRH pulse. To gain insight into the possible differential effects of T on the two kinetic phases of the GTH-II response, the "peak" and "plateau" responses to the first GnRH challenges were also analyzed.

The presence of T significantly potentiated the GTH-II response to sGnRH in both the "peak" and "plateau" phases in all three gonadal maturational stages (Tables III.1A and B). In the "peak" phase of the sGnRH-elicited GTH-II response,

the magnitude of the response increased with increasing gonadal maturity in control and T-treated cells (Table III.1A). A similar effect was also seen in the "plateau" phase of the sGnRH-induced GTH-II response (Table III.1B). Correspondingly, the duration of the sGnRH-induced GTH-II response also increased as the gonads progressed from regressed to mature (regressed: 9.0 ± 3.5 min; recrudescing: 16.5 ± 3.8 min; mature: 26.0 ± 4.2 min; $p<0.05$ prespawning vs. earlier stages of ovarian maturation; Dunn's test). These results indicate the potentiating actions of T on either phases of the sGnRH-induced GTH-II response were more prominent in cells from later stages of gonadal maturation.

In control cells, the cGnRH-II-induced GTH-II response in the "peak" and "plateau" phases were comparable in cells from sexually regressed, recrudescing, and prespawning female goldfish (Tables III.2A and B). T treatments significantly enhanced the cGnRH-II-induced GTH-II responses in the "peak" and "plateau" phases in all three gonadal maturational stages (Tables III.2A and B). In contrast to sGnRH, increasing gonadal maturity of the pituitary donor affected the potentiating actions of T on the cGnRH-II-stimulated GTH-II response in the "peak" phase, but not in the "plateau" phase (Tables III.2A and B). In addition, the duration of the release responses were also not affected by ovarian conditions (regressed: 16.0 ± 1.8 min; recrudescence: 18.5 ± 6.3; mature: 19.0 ± 2.1 min; $p>0.05$ vs. one another, Kruskal Wallis test).

In cells from prespawning females alone and from prespawning male and female goldfish, the total GnRH-induced response as well as the size of both the "peak" and "plateau" GTH-II response phases to the native GnRHs in experiments were similar at each of the T treatment doses (Figs. 3, 6, 7, 8; Table III.1-3). These results indicate that T pretreatment was as effective in enhancing the two kinetic phases of the GTH-II responses to sGnRH and cGnRH-II in pituitary cell cultures from males and females, as in cells from female goldfish alone. Thus, cells from both male and

female goldfish were used in subsequent experiments for investigating the actions of T on GnRH-stimulated GTH-II release.

III.2. Effects of T on the dose-dependent GTH-II release response to sGnRH and cGnRH-II.

Previous studies have demonstrated that sGnRH and cGnRH-II increased GTH-II release from dispersed goldfish pituitary cells in a dose-dependent manner with ED_{50}s in the nM range and with maximal stimulatory effects observed at 100 nM GnRH (Chang *et al*, 1990a). In the present study with dispersed pituitary cells obtained from sexually regressed male and female goldfish, perifusion with 0.1 to 100 nM doses of either sGnRH or cGnRH-II increased GTH-II release in control cells not pretreated with additional T (Fig. 9; responses to all doses of GnRH significantly greater than zero; $p<0.05$; $n = 10$). The estimated ED_{50}s of the sGnRH- and cGnRH-II-stimulated GTH-II responses were 12.2 ± 2.8 and 2.0 ± 0.4 nM, respectively. In cells pretreated with either 10 or 100 nM T, all doses of GnRH tested effectively increased GTH-II release (Fig. 9; responses to all doses of GnRH significantly greater than zero; $p<0.05$; $n = 10$). Assuming that the maximal effective GnRH dose remained at 100 nM in T-pretreated cells, the estimated ED_{50}s for sGnRH- and cGnRH-II-induced GTH-II release were 22.1 ± 1.5 and 9.8 ± 2.9 nM in 10 nM T-pretreated cells, and 15.1 ± 2.3 and 17.2 ± 2.9 nM in 100 nM T-pretreated cells, respectively. Considering the 95% confidence limits of these values, treatment with 10 nM T significantly increased the ED_{50} values for the sGnRH-induced GTH-II; whereas both 10 and 100 nM T elevated the ED_{50} values for cGnRH-II-induced GTH-II release. Similarly, treatment with 10 and 100 nM T significantly elevated the maximal GTH-II responses to sGnRH and cGnRH-II stimulation. (Maximal response estimates in control, 10 nM T- and 100 nM T-treated groups were 106 ± 5, 472 ± 7 and 508 ± 12%

pretreatment for sGnRH, and 122 ± 3.5, 391 ± 27 and 487 ± 17% pretreatment for cGnRH, respectively; 95% confidence intervals of values in T-treated cells not overlapping with those for controls.) Thus, pretreatment with T enhanced the efficacy of the GTH-II releasing ability of the two native GnRHs with a decrease in the potency of the GnRH dose-response curves. Since the lowest GnRH dose tested (0.01 nM) already elevated GTH-II secretion significantly in control cells, whether the sensitivity to GnRH (minimal effective dose) was altered by pretreatment with T could not be determined.

III.3. Effects of T on pituitary cellular GTH-II contents and responsiveness to GnRH in static culture.

To examine the possibility that T could increase the availability of GTH-II protein contents, the effects of T pretreatment on cellular GTH-II contents and GnRH-induced GTH-II secretion were monitored in static incubation studies. Static incubation experiments enable the easy harvesting of pituitary cells following drug treatment.

III.3.1. *Short-term (0.5 h) GnRH treatment.*

In the first series of experiments, dispersed pituitary cells obtained from sexually regressed male and female goldfish were treated with 10 nM T for 28 h in static incubation followed by a 0.5-h GnRH challenge. These durations of drug treatment mimic those used in previous perifusion studies. 10 nM T was used because this concentration is physiological and has been shown to be effective in most of the earlier experiments.

During the 28-h incubation period, no significant differences were observed between the T-treated and untreated cells in terms of the amount of GTH-II released, the remaining cellular GTH-II contents, and the total GTH-II measured (Fig. 10). These results indicate that basal GTH-II release was not altered by T-treatment during this period, and that T did not increase the total production of radioimmunoassayable GTH-II. Thus, both the untreated and T-treated cells had a similar amount of radioimmunoassayable GTH-II available before the 0.5-h GnRH challenge.

A 100 nM 0.5-h sGnRH or cGnRH-II treatment led to a significant increase in GTH-II release in T-treated but not in untreated cells (Fig. 11A). This suggests that T pretreatment enhanced the GTH-II release responses to both native GnRHs in static incubation studies as in perifusion experiments. GnRH treatment did not significantly reduce the amount of GTH-II remaining in the cell but a difference was detected between T-treated and untreated cells in the group exposed to sGnRH, probably due to increased GTH-II release (Fig. 11B). Nevertheless, the total GTH-II contents were similar between GnRH-stimulated and unstimulated cells regardless of T exposure (Fig. 11C). This suggests that GnRH is unlikely to have caused any GTH-II synthesis during this 0.5-h incubation and that T pretreatment has no effects on this process.

In these experiments, significant GTH-II release responses to sGnRH and cGnRH-II could not be detected in untreated cells. One possible explanation is that the increase in GnRH-stimulated GTH-II release was too small in static incubation studies to be detected during the 0.5-h treatment because of the relatively high basal GTH-II values as compared to perifusion experiments. Thus, a longer period of GnRH treatment, such as those used in other routine static incubation experiments (2 h; Chang *et al*, 1990a), was used in subsequent experiments to further investigate if T could affect GnRH actions on GTH-II release and cellular GTH-II contents.

III.3.2. *Long-term (2 h) GnRH treatment.*

The effects of 10 nM T on GTH-II release and contents were investigated in pituitary cells prepared from male and female goldfish undergoing sexual recrudescence after 24 h of incubation and a 2-h GnRH treatment in static culture. During the 24-h incubation, exposure to 10 nM T did not alter the amount of GTH-II released, the remaining GTH-II contents, and the total GTH-II contents (Fig. 12). These results suggest that T treatment affected neither basal GTH-II release over the 24-h incubation period, the production of radioimmunoassayable GTH-II, nor the amount of radioimmunoassayable GTH-II available within cells prior to the subsequent GnRH challenge.

During the 2-h GnRH treatment, 100 nM cGnRH-II, but not 100 nM sGnRH, significantly increased in GTH-II release in untreated cells (Fig. 13A). In T-treated cells, both sGnRH and cGnRH-II induced an increase in GTH-II release. In addition, the GTH-II responses to both GnRHs were greater than those observed in untreated cells (Fig. 13A). Thus, T is also effective in potentiating GnRH-stimulated GTH-II release from dispersed goldfish pituitary cells in 2-h incubation studies as in perifusion experiments.

The amount of GTH-II remaining in the pituitary cells after the 2-h GnRH treatment is shown in Figure 13B. GnRH treatment did not affect the amount of GTH-II remaining in either T-treated or untreated cells. However, the remaining GTH-II content was lower in the T-treated group exposed to sGnRH than in the sGnRH-exposed group not treated with T. On the other hand, the total GTH-II contents measured were not different among all the treatment groups (Fig. 13C). This suggests that neither GnRHs significantly affected the production of total radioimmunoassayable GTH-II content during a 2-h challenge, and that T pretreatment had no influence on these processes.

These results with static incubation studies (Figs. 10-13) strongly indicate that pre-exposure of cells to T did not lead to a significant increase in cellular GTH-II content either before or after GnRH treatments. Moreover, this suggests that the observed increase in GTH-II release in T-treated cells is not due to elevations in GTH-II protein synthesis either before or during the GnRH challenge. It is possible that T potentiates GnRH-stimulated GTH-II release by modulating the GnRH signal transduction pathway.

III.4. Effects of T on the effectiveness of signal transduction components mediating GnRH action.

Previous experiments have indicated that PKC activation and extracellular Ca^{2+} entry through VSCC are important components of the signal transduction pathways for both sGnRH and cGnRH-II in stimulating GTH-II secretion (reviewed in Chang *et al*, 1996; see section I.3). The possible effects of T treatment on the effectiveness of these two signalling pathways were investigated.

III.4.1. *Involvement of PKC.*

The effects of T-treatment on PKC-induced GTH-II release were first examined. Dispersed pituitary cells obtained from male and female goldfish during the prespawning season were incubated in either 0, 1, or 10 nM T for 24 h and then treated with 0.01 to 100 nM TPA, an activator of PKC, in 2-h static incubation studies. T-treatment decreased basal GTH-II release in these experiments (Table III.4). In untreated cells, 10 and 100 nM TPA were effective in inducing a GTH-II response ($p<0.05$ vs. 0 nM TPA). Pituitary cells treated with 1 nM T had a significant GTH-II response to 10 nM TPA ($p<0.05$ vs. 0 nM TPA). In 10 nM T-treated cells, 1, 10, and

100 nM TPA significantly elevated GTH-II secretion ($p<0.05$ vs. 0 nM TPA). The estimated ED_{50}s of the GTH-II dose-response curve to TPA in 0, 1, and 10 nM T-treated cells were not different from one another, being 0.8 ± 20.7, 0.1 ± 0.9, and 0.2 ± 0.4 nM, respectively. The minimum effective doses of TPA required to significantly stimulate GTH-II response in 0, 1, and 10 nM T-treated cells were 10, 10, and 1 nM TPA, respectively (Table III.1). This suggests that the sensitivity of the pituitary cells to TPA stimulation has shifted with T treatment. To estimate if the efficacy of TPA-induced GTH-II secretion was altered, the net GTH-II response to TPA was calculated. This corrects for the significant differences in unstimulated GTH-II release between controls and the T-treated groups. The net GTH-II response to maximal (100 nM) TPA in cells pretreated with 10 nM T-treated, but not that in the 1 nM T-pretreated group, was significantly elevated compared to the response observed in controls (Table III.4). The net GTH-II responses to submaximal doses of TPA (0.1 to 10 nM) were also enhanced in 10 nM T-treated cells (Table III.4). From these experiments, it is demonstrated that 10 nM T enhances PKC activator-induced GTH-II response by increasing the sensitivity of the response and the efficacy of the stimulation.

To evaluate the possible participation of PKC-dependent GTH-II release in the potentiating effects of T on GnRH-induced GTH-II response, the effects of T pretreatment on the ability of a PKC inhibitor, H7, to decrease TPA- and GnRH-stimulated GTH-II release were investigated. Neither pretreatment with 10 nM T nor exposure to 10 μM H7 alone altered basal GTH-II release (Figs. 14 and 15). TPA, at a dose of 100 nM, was effective in inducing GTH-II release from both untreated and T-treated cells; however, pretreatment with T significantly enhanced the GTH-II response to 100 nM TPA above the response in control cells (Fig. 14). Pretreatment with 10 nM T elevated the GTH-II response to both GnRHs above those observed in control cells (Fig. 15) as in previous experiments. Application of 10 μM H7 partially reduced the GTH-II responses to TPA in both T-treated and untreated cells, and abolished those to

either native GnRHs regardless of T pretreatment (Figs. 14 and 15). Taken together, these results suggest that PKC-dependent mechanisms are possible targets of T action. Results further indicate that the potentiating actions of T on GnRH-stimulated GTH-II release is likely dependent on the presence of PKC.

III.4.2. *Ca^{2+}-dependent GTH-II exocytosis.*

GnRH-induced GTH-II secretion has been shown to be a highly Ca^{2+}-dependent process (see section I.3.3). The possible influence of T pretreatment on Ca^{2+}-dependent GTH-II exocytosis was investigated using a Ca^{2+}-ionophore, ionomycin, in static incubation studies with pituitary cells prepared from male and female goldfish in the postspawning season. In untreated cells, 5, 10 and 100 μM ionomycin were all effective in stimulating GTH-II release (Table III.5). These same doses of ionomycin also induced GTH-II release in 10 nM T-treated cells (Table III.5). Therefore, the minimum effective dose of ionomycin in stimulating GTH-II release was 5 μM in both untreated and T-treated cells. T pretreatment did not affect the estimated ED_{50}s values of the GTH-II dose-response curve to ionomycin (ED_{50} = 7.8 ± 2.8 and 8.7 ± 3.0 μM in control and T-treated cells, respectively; $p>0.05$ vs. one another). Since basal GTH-II secretion was significantly reduced by T pretreatment in these experiments, the net responses to the maximal 100 μM ionomycin were also calculated as an evaluation of the efficacy of the ionophore-stimulated release. Preexposure to T did not alter the net GTH-II response to 100 μM ionomycin compared to the response observed in control cells (Table III.5). Thus, T-treatment altered neither the efficacy, the sensitivity, nor the potency of the ionomycin stimulation of GTH-II secretion. This suggests that T pretreatment has no effects on Ca^{2+}-dependent GTH-II exocytosis or the total Ca^{2+}-releasable GTH-II pool.

III.4.3. *Extracellular Ca^{2+} entry via voltage-sensitive Ca^{2+} channel.*

The possibility that T affects on GnRH-induced GTH-II release were expressed by action at the level of VSCC to enhance Ca^{2+} influx was investigated. This was examined indirectly by studying the effects of T on the GTH-II response to a VSCC agonist, Bay K 8644. Pituitary cells obtained from sexually regressed male and female goldfish were used. Pretreatment with 10 nM T did not affect basal GTH-II release. In control and T-treated cells, 10 μM Bay K 8644 stimulated GTH-II release (Fig. 16). Potency estimates (ED_{50}s) for Bay K 8644 were not performed as too few doses of the VSCC agonist effectively stimulated GTH-II release. Since the maximal dose of Bay K 8644 used (10 μM) was equally effective in increasing the GTH-II response in untreated and T-treated cells (Fig. 16), these results suggest that T pretreatment did not alter the efficacy of Bay K 8644 in inducing GTH-II secretion. Sensitivity to Bay K 8644 stimulation was not altered after T treatment since the minimum effective dose for both control and T-treated cells was 10 μM.

III.4.4. *Extracellular Ca^{2+}-dependent GnRH action on GTH-II release.*

It has been demonstrated that, in goldfish, GnRH action on GTH-II release is dependent on the entry of extracellular Ca^{2+} through VSCC (see section I.3.3). To examine the possible involvement of VSCC in GnRH action following T-pretreatment, the effects of a VSCC inhibitor, nifedipine, on the GTH-II responses to 100 nM GnRH pulses were examined in cell column perifusion experiments. Nifedipine was applied during the second of three sequential applications of 5-min pulses of 100 nM GnRH, separated by 1-h intervals. This protocol of GnRH application has been shown to produce repeatable GTH-II release responses in non T-treated cells (Jobin *et al*, 1996a). Likewise, 3 sequential applications of 5-min GnRH

pulses produced 3 similar GTH-II responses in 10 nM T-treated cells (2 columns for each sGnRH and cGnRH-II treatment, data not shown).

III.4.4.1. sGnRH-stimulated GTH-II release.

Pituitary cells prepared from sexually regressed male and female goldfish were used in these experiemnts. In control cells not pretreated with T, application of a 5-min pulse of 100 nM sGnRH stimulated GTH-II secretion prior to the application of the organic VSCC blocker (pretest; Figs. 17A and B). Application of 1 μM nifedipine reduced the GTH-II response to sGnRH (Fig. 17B). Following the removal of nifedipine (washout), the response to sGnRH returned to a level not different from that obtained prior to nifedipine application (Fig. 17B). Prior exposure to nifedipine alone also did not affect the magnitude of the GTH-II respones to a subsequent sGnRH challenge (Fig. 17A).

Pretreatment with 10 nM T did not alter basal GTH-II release rate but significantly increased the sGnRH-induced GTH-II response during the pretest period compared to control cells (Figs. 17A and B). In T-treated cells, application of nifedipine reduced the GTH-II response to sGnRH compared to the pretest response; however, the hormone release respone did not recover upon the removal of nifedipine (Fig. 17B). The response observed following washout was significantly smaller than that observed during the pretest period. Prior exposure to nifedipine alone also reduced the GTH-II response to sGnRH in T-treated cells (Fig. 17A). In all test periods, the sGnRH-induced GTH-II responses remained higher in T-treated cells than in control cells.

These observations indicate that T pretreatment increased the GTH-II response to a 5-min pulse of sGnRH as in previous experiments with 30-min pulses of

sGnRH. The results also suggest that a VSCC-dependent component exists in sGnRH stimulation of GTH-II release in both T-pretreated and untreated cells.

III.4.4.2. cGnRH-II-stimulated GTH-II release.

Pituitary cells prepared from sexually regressed male and female goldfish were used in these experiments. In control cells, application of 10 μM nifedipine reduced the 100 nM cGnRH-II-stimulated GTH-II response. The GTH-II response to cGnRH-II was significantly lower in the presence of nifedipine than that in the pretest preiod. (Fig. 18B). Following the removal of nifedipine (washout), the response to cGnRH-II recovered completely, to a level significantly higher than that observed during nifedipine application and not different from the pretest response (Fig. 18B). Prior exposure to nifedipine alone also did not affect the magnitude of the GTH-II response to a subsequent cGnRH-II challenge (Fig. 18A).

Pretreatment with 10 nM T did not alter basal GTH-II release rate but significantly increased the cGnRH-II-induced GTH-II release above those observed in control cells in all test periods (Figs. 18A and B). In T-treated cells, application of nifedipine reduced the GTH-II response to cGnRH-II compared to the pretest response (Fig. 18B). However, unlike control cells, the hormone release response remained suppressed in T-treated cells even following the removal of nifedipine (Fig. 18B). Prior exposure to nifedipine alone also reduced the GTH-II response to cGnRH-II in T-pretreated cells (Fig. 18A).

These observations indicate that T-pretreatment increased the GTH-II response to a 5-min pulse of cGnRH-II as in previous experiments with 30-min pulses of cGnRH-II. The results also suggest that a VSCC-dependent component exists in cGnRH-II stimulation of GTH-II release in both T-pretreated and untreated cells.

III.5. Involvement of aromatization in the actions of T on GnRH-stimulated GTH-II release.

III.5.1. *Effects of nonaromatizable androgens on GnRH-stimulated GTH-II release.*

As described in the Introduction (section I.2.5), many of the actions of T are mediated via its conversion to nonaromatizable metabolites or aromatization to E_2. To examine these possibilities, the effects of nonaromatizable androgens on 100 nM sGnRH-stimulated GTH-II release were first examined in cell column perifusion studies. Pituitary cells prepared from male and female goldfish undergoing gonadal recrudescence were used in these experiments.

Pretreatment with 10 nM T enhanced the GTH-II responses to the first 0.5-h pulse application of sGnRH compared to controls (Fig. 19). In contrast, pretreatment with either 10 nM 11-KT or 10 nM DHT did not alter the sGnRH-stimulated GTH-II response as compared to that from untreated cells (Fig. 19). Compared to the responses observed in the first GnRH pulse, the responses to the second GnRH pulse were similar in magnitude in the controls, 11-KT and DHT-treated cells. The second sGnRH-stimulated GTH-II response in cells exposed to 10 nM T was significantly reduced compared to the response to the first sGnRH challenge; furthermore, it was not significantly elevated compared to control values (Fig. 19). These results demonstrate that the effects of T on potentiating GnRH-induced GTH-II release are not mimicked by its nonaromatizable metabolites.

III.5.2. *Effect of an aromatase inhibitor, ATD, on the potentiating actions of T on GnRH-stimulated GTH-II release.*

The possible involvement of aromatization in mediating the potentiating effects of 10 nM T on 100 nM GnRH-stimulated GTH-II release from goldfish pituitary cells was examined in 2-h static incubation studies using the aromatase inhibitor, ATD (300 μM). Dispersed pituitary cells obtained from prespawning male and female goldfish were used in these experiments. In untreated cells, the GTH-II responses to sGnRH and cGnRH-II were not significantly elevated above control values (Fig. 20A). On the other hand, both sGnRH and cGnRH-II significantly elevated GTH-II release in T-treated cells. These GnRH-induced GTH-II responses in the T-treated cells were also significantly higher than those in untreated cells (Fig. 20A). Treatment with ATD alone did not affect GTH-II release from unstimulated or GnRH-stimulated cells (Fig. 20A). But in the presence of T pretreatment, ATD abolished the GnRH-induced GTH-II release (Fig. 20A). These results indicate that ATD abolished the ability of T treatment to increase the GTH-II response to stimulation by sGnRH and cGnRH-II.

The effects of ATD on total GTH-II contents were also examined. In untreated cells, no significant difference was observed between the unstimulated and GnRH-stimulated groups (Fig. 20B). The total GTH-II contents did not differ between the unstimulated and GnRH-stimulated groups in the cells pretreated with T (Fig. 20B). In addition, these total GTH-II contents were similar to those in untreated cells (Fig. 20B). This indicates that T-treatment did not alter the total GTH-II contents as has been shown in earlier experiments reported in this thesis. In general, cells treated with ATD tended to have higher total GTH-II contents. In sGnRH-stimulated cells, addition of ATD alone increased total GTH-II contents significantly above those of untreated and T-treated cells. Blockade of aromatase activity may have a positive influence over total GTH-II contents under some conditions.

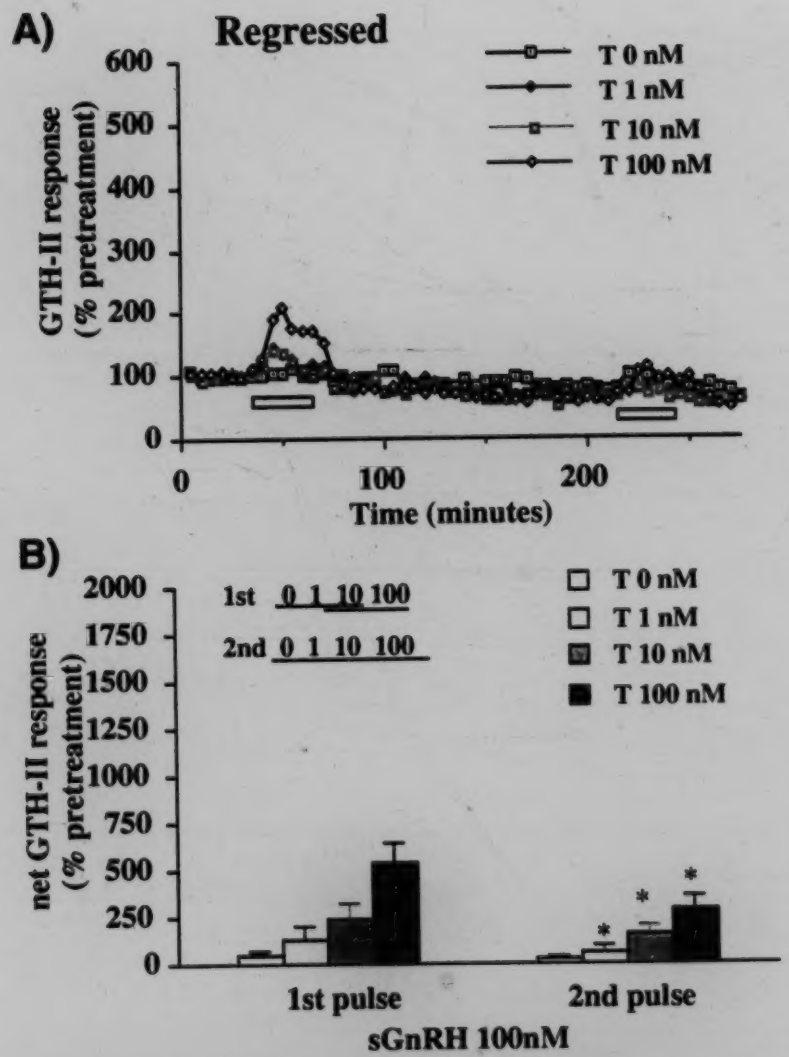

Figure 1. Effects of different doses of T on 100 nM sGnRH-stimulated GTH-II release from dispersed pituitary cells obtained from sexually regressed female goldfish (GSI = 1.3 ± 0.2%). A) Examples of GTH-II release profiles are presented. The open bars indicate the two 0.5-h 100 nM sGnRH pulses. B) Net sGnRH-induced GTH-II responses (mean ± SEM) calculated from pooled data. Average pretreatment GTH-II values for control, 1, 10, and 100 nM T-treated columns were similar, being 3.1 ± 0.3, 2.7 ± 0.3, 2.3 ± 0.2, and 2.8 ± 0.1 ng/ml respectively (n = 4-6 columns). T treatments resulting in similar GTH-II responses share the same underscore (Kruskal Wallis test followed by Dunn's test, $p>0.05$). * denotes significant difference between the GTH-II response to the first and second sGnRH pulse ($p<0.05$; n = 4-6; Wilcoxon signed-rank test).

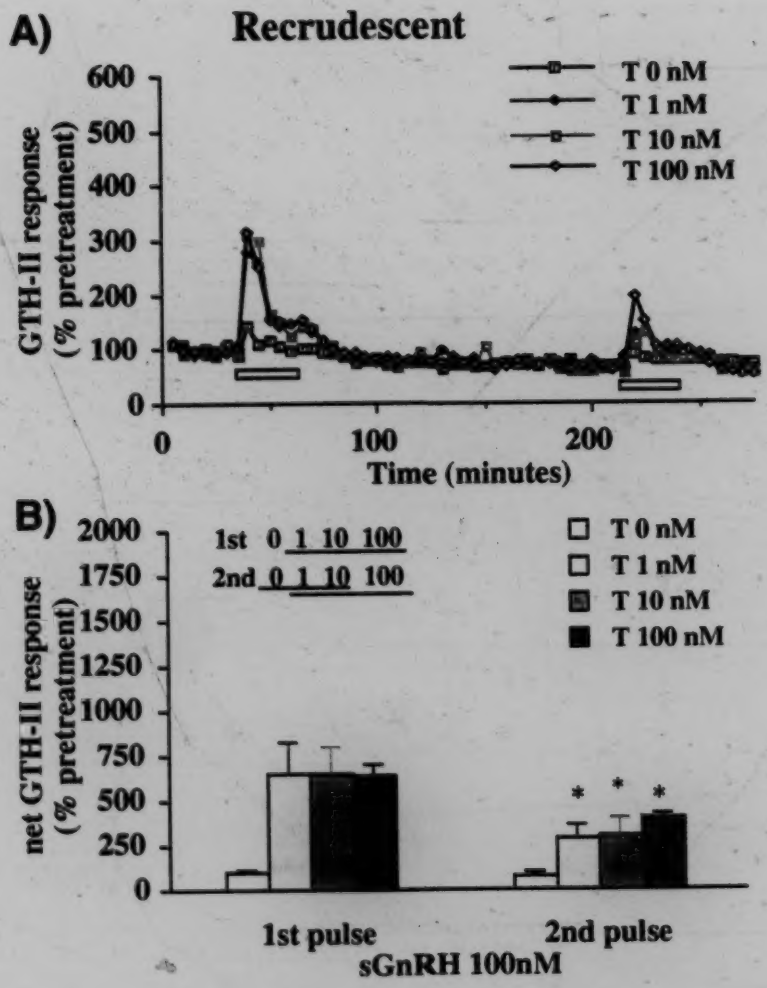

Figure 2. Effects of different doses of T on 100 nM sGnRH-stimulated GTH-II release from dispersed pituitary cells obtained from sexually recrudescent female goldfish (GSI = 3.4 ± 1%). A) Examples of GTH-II release profiles are presented. The open bars represent the two 0.5-h 100 nM sGnRH pulses. B) Net GTH-II responses (mean ± SEM) to 100 nM sGnRH calculated from pooled data. Average pretreatment GTH-II values for control, 1, 10, and 100 nM T-treated columns were similar, being 3.7 ± 0.3, 3.1 ± 0.5, 3.2 ± 0.6, and 4.0 ± 0.1 ng/ml, respectively (n = 6-8 columns). T treatments resulting in similar GTH-II responses were joined by the same underscore (Kruskal Wallis test followed by Dunn's test, $p>0.05$). * denotes significant difference between the GTH-II response to the first and second sGnRH pulse ($p<0.05$; n= 6-8; Wilcoxon signed-rank test).

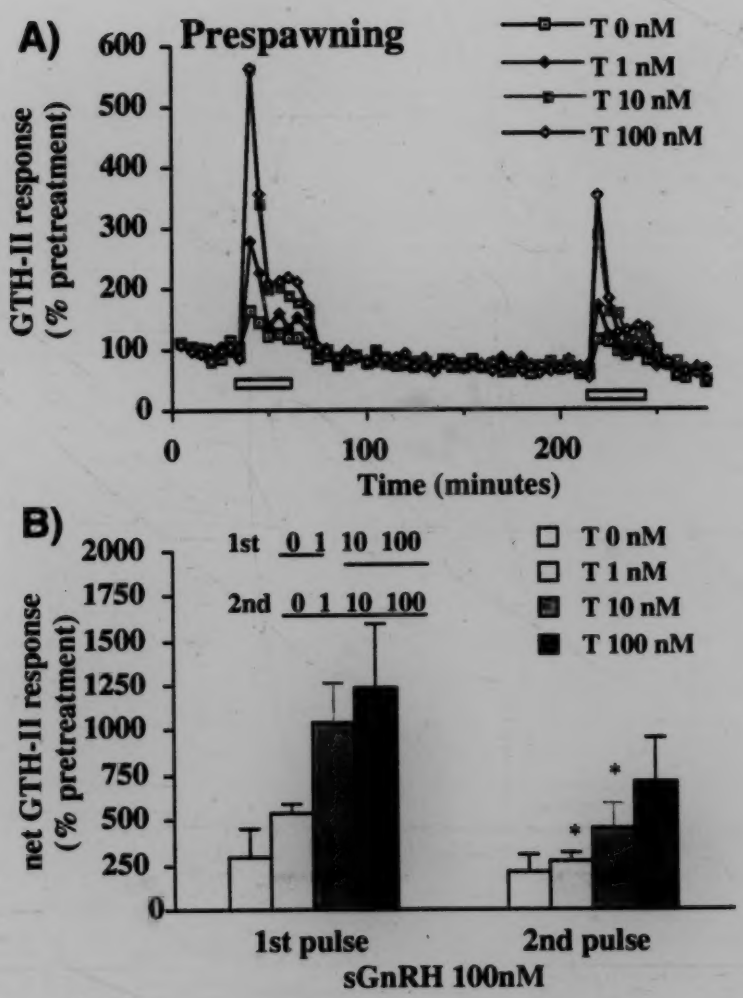

Figure 3. Effects of different doses of T on 100 nM sGnRH-stimulated GTH-II release from dispersed pituitary cells obtained from prespawning female goldfish (GSI = 8.3 ± 1.4%). A) Examples of GTH-II release profiles are presented. The open bars represent the two 0.5-h 100 nM sGnRH pulses. B) Net GTH-II response to 100 nM sGnRH (mean ± SEM) calculated from pooled data. Average pretreatment GTH-II values for control, 1, 10, and 100 nM T-treated columns were similar, being 2.0 ± 0.3, 1.9 ± 0.2, 1.3 ± 0.2, and 1.8 ± 0.1 ng/ml, respectively (n = 6-8 columns). T treatments resulting in similar GTH-II responses share the same underscore (Kruskal Wallis test followed by Dunn's test, $p>0.05$). * denotes significant difference between the GTH-II response to the first and second sGnRH pulse ($p<0.05$; n= 6-8; Wilcoxon signed-rank test).

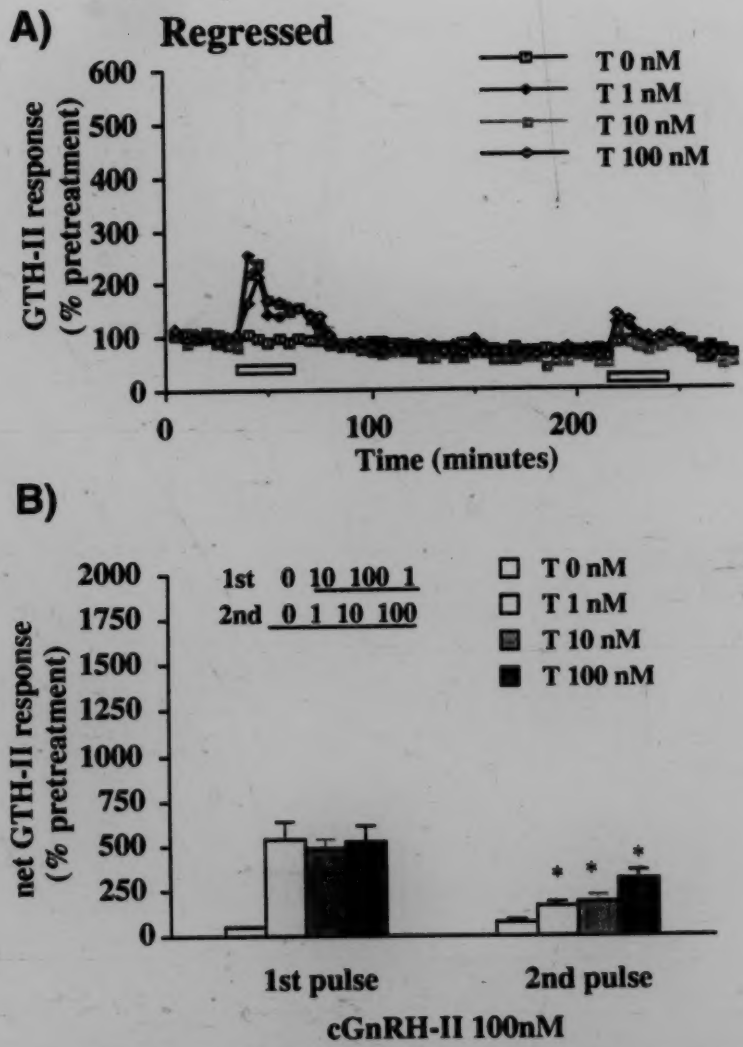

Figure 4. Effects of different doses of T on 100 nM cGnRH-II-stimulated GTH-II release from dispersed pituitary cells obtained from sexually regressed female goldfish (GSI = 1.0 ± 0.3%). A) Examples of GTH-II release profiles are presented. The open bars represent the two 0.5-h 100 nM cGnRH-II pulses. B) Net GTH-II responses to 100 nM cGnRH-II (mean ± SEM) calculated from pooled data. Average pretreatment GTH-II values for control, 1, 10, and 100 nM T-treated columns were similar, being 3.3 ± 0.5, 2.7 ± 0.3, 3.5 ± 0.3, and 2.5 ± 0.2 ng/ml, respectively (n = 6 columns). T treatments resulting in similar GTH-II responses share the same underscore (Kruskal Wallis test followed by Dunn's test, $p>0.05$). * denotes significant difference between the GTH-II response to the first and second cGnRH-II pulse ($p<0.05$; n= 6; Wilcoxon signed-rank test).

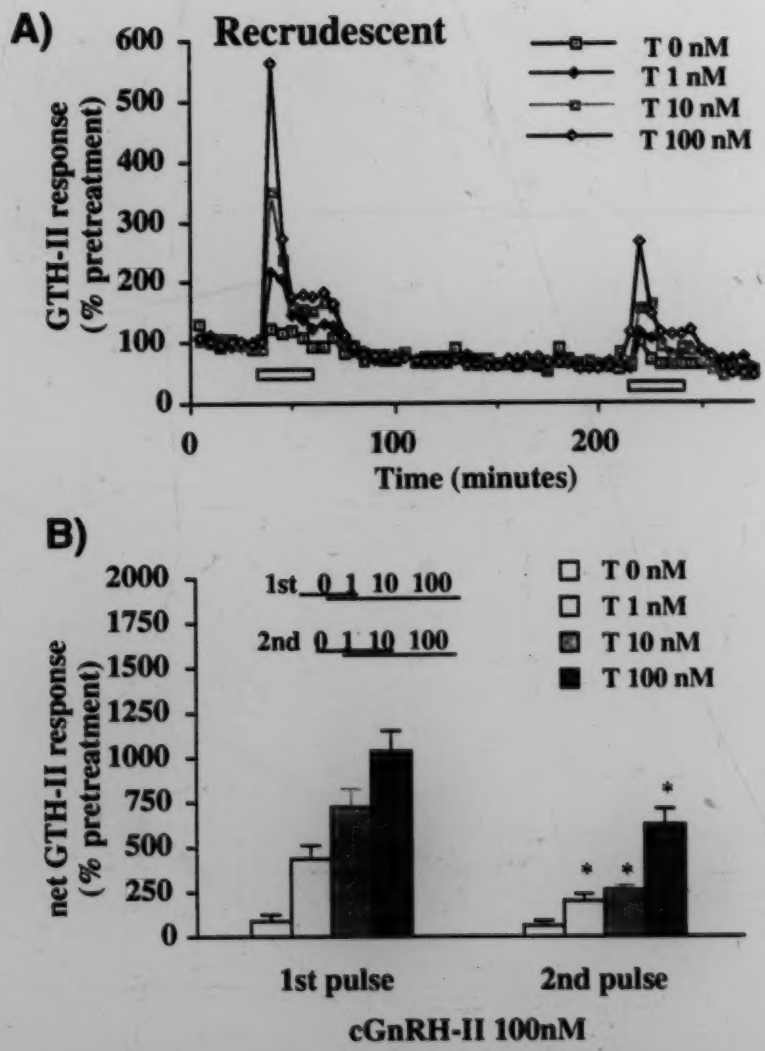

Figure 5. Effects of different doses of T on 100 nM cGnRH-II-stimulated GTH-II release from dispersed pituitary cells obtained from sexually recrudescent female goldfish (GSI = 4.8 ± 0.8%). A) Examples of GTH-II release profiles are presented. The open bars represent the two 0.5-h 100 nM cGnRH-II pulses. B) Net GTH-II responses to 100 nM cGnRH-II (mean ± SEM) calculated from pooled data. Average pretreatment GTH-II values for control, 1, 10, and 100 nM T-treated columns were similar, being 4.3 ± 0.3, 3.4 ± 0.3, 3.2 ± 0.3, and 4.5 ± 0.2 ng/ml, respectively (n = 6-8 columns). T treatments resulting in similar GTH-II responses share the same underscore (Kruskal Wallis test followed by Dunn's test, $p>0.05$). * denotes significant difference between the GTH-II response to the first and second cGnRH-II pulse ($p<0.05$; n= 6-8; Wilcoxon signed-rank test).

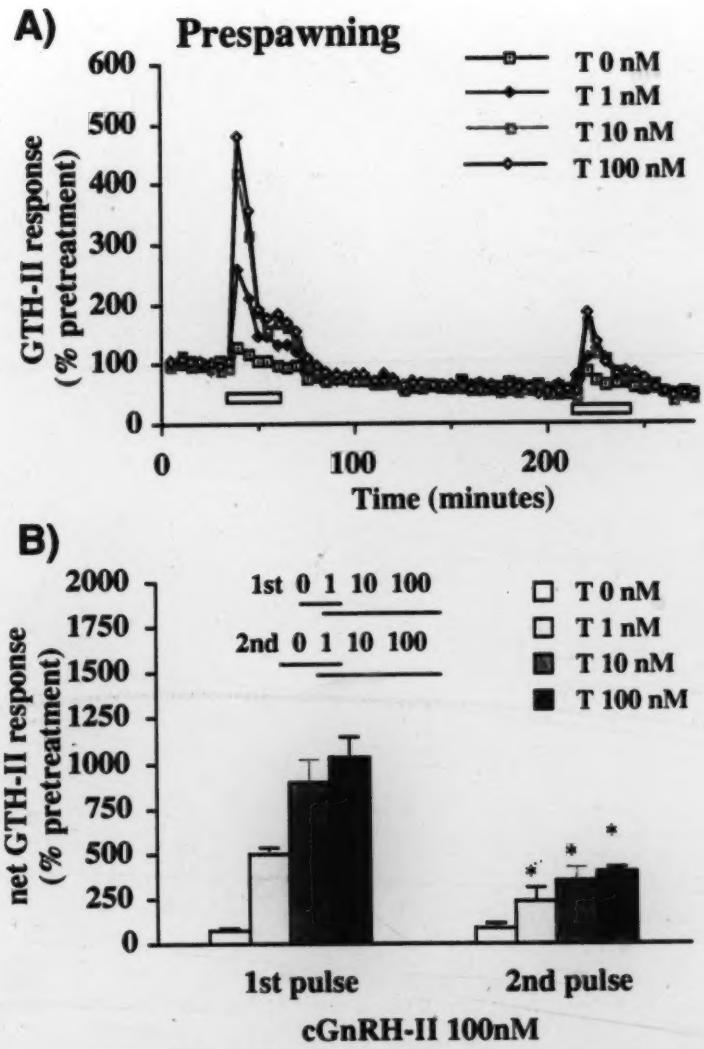

Figure 6. Effects of different doses of T on 100 nM cGnRH-II-stimulated GTH-II release from dispersed pituitary cells obtained from prespawning female goldfish (GSI = 8.1 ± 0.9%). A) Examples of GTH-II release profiles are presented. The open bars represent the two 0.5-h 100 nM cGnRH-II pulses. B) Net GTH-II responses to 100 nM cGnRH-II (mean ± SEM) calculated from pooled data. Average pretreatment GTH-II values for control, 1, 10, and 100 nM T-treated columns were similar, being 3.5 ± 0.3, 2.6 ± 0.2, 2.8 ± 0.2, and 2.6 ± 0.1 ng/ml, respectively (n = 6 columns). T treatments resulting in similar GTH-II responses share the same underscore (Kruskal Wallis test followed by Dunn's test, $p>0.05$). * denotes significant difference between the GTH-II response to the first and second cGnRH-II pulse ($p<0.05$; n = 6; Wilcoxon signed-rank test).

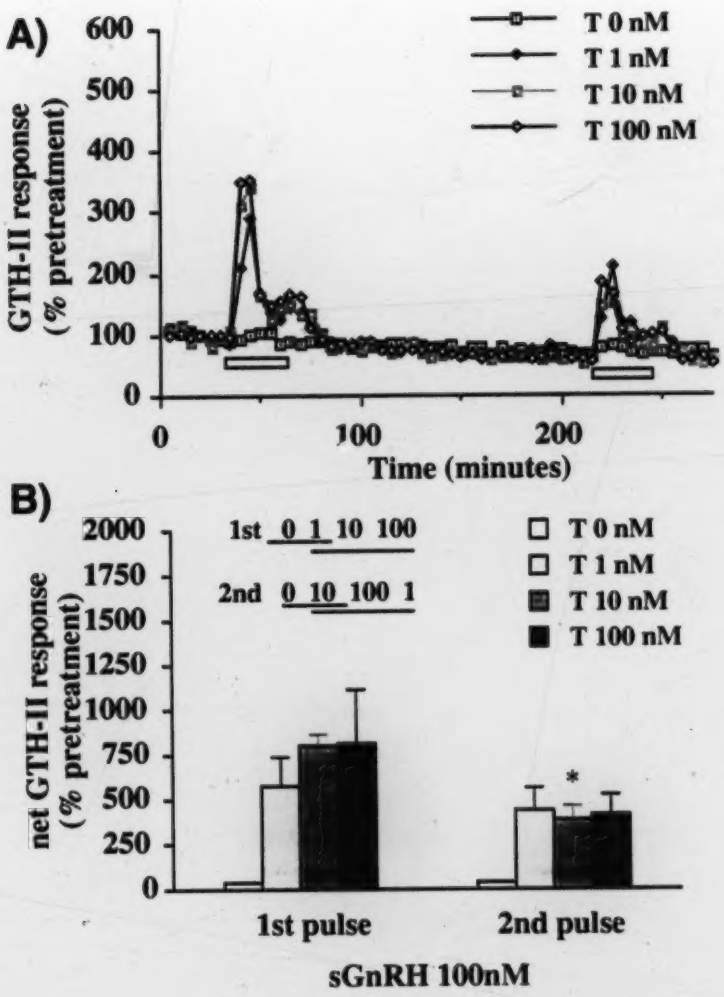

Figure 7. Effects of different doses of T on 100 nM sGnRH-stimulated GTH-II release from dispersed pituitary cells obtained from prespawning male and female goldfish (GSI = 7.5 ± 1.2%). A) Examples of GTH-II release profiles are presented. The open bars represent the two 0.5-h 100 nM sGnRH pulses. B) Net GTH-II response to 100 nM sGnRH (mean ± SEM) calculated from pooled data. Average pretreatment GTH-II values for control, 1, 10, and 100 nM T-treated columns were similar, being 5.0 ± 0.3, 5.0 ± 0.2, 4.3 ± 0.3, and 4.7 ± 0.2 ng/ml, respectively (n = 4 columns). T treatments resulting in similar GTH-II responses share the same underscore (Kruskal Wallis test followed by Dunn's test, $p>0.05$). * denotes significant difference between the GTH-II response in the first and second sGnRH pulses ($p<0.05$; n = 4; Wilcoxon signed-rank test).

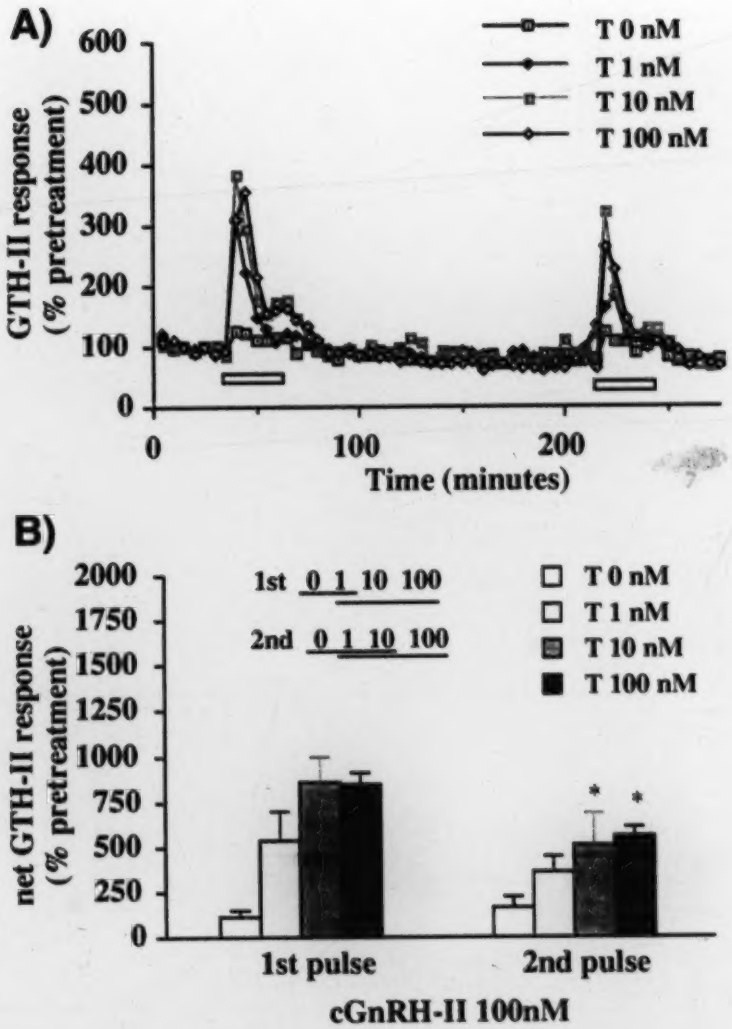

Figure 8. Effects of different doses of T on 100 nM cGnRH-II-stimulated GTH-II release from dispersed pituitary cells obtained from prespawning male and female goldfish (GSI = 8.2 ± 1.7%). A) Examples of GTH-II release profiles are presented. The open bars represent the two 0.5-h 100 nM cGnRH-II pulses. B) Net GTH-II response to 100 nM cGnRH-II (mean ± SEM) calculated from pooled data. Average pretreatment GTH-II values for control, 1, 10, and 100 nM T-treated columns were similar, being 3.6 ± 0.5, 4.1 ± 0.3, 3.8 ± 0.2, and 3.4 ± 0.2 ng/ml, respectively (n = 4 columns). T treatments resulting in similar GTH-II responses share the same underscore (Kruskal Wallis test followed by Dunn's test, $p>0.05$). * denotes significant difference between the GTH-II response in the first and second cGnRH-II pulses ($p<0.05$; n = 4; Wilcoxon signed-rank test).

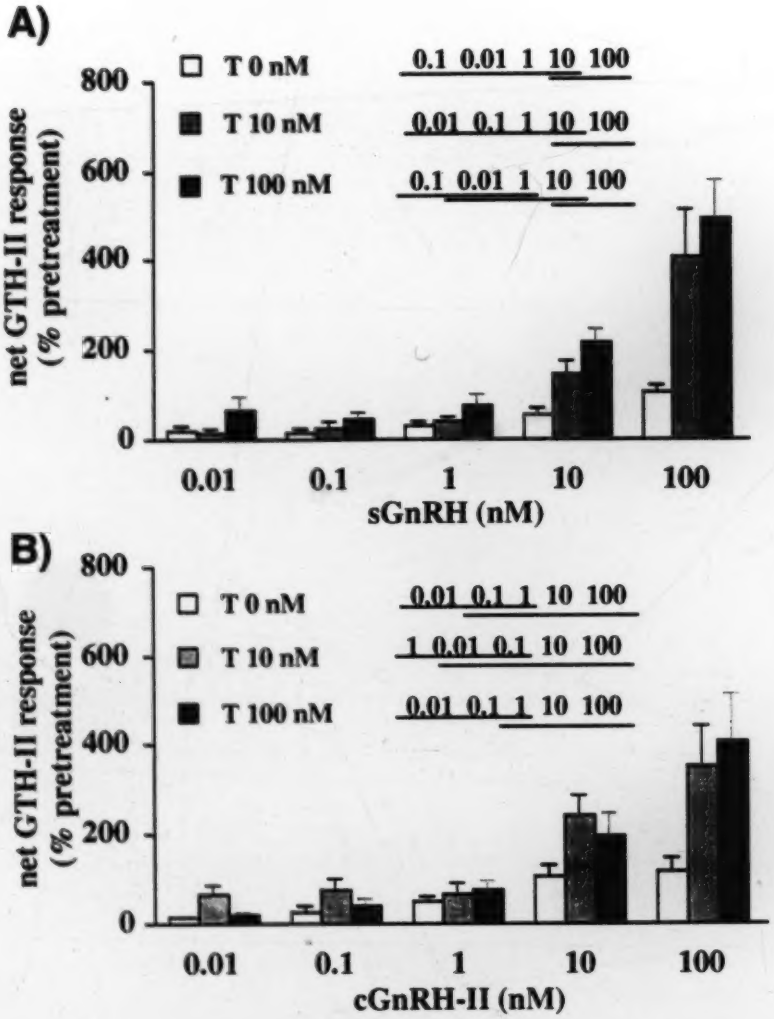

Figure 9. Effects of T on the net GTH-II response (mean ± SEM) to different doses of A) sGnRH and B) cGnRH-II in cell column perifusion experiments with pituitary cells obtained from sexually regressed male and female goldfish. Average pretreatment values for sGnRH experiments in control, 10, and 100 nM T-treated cells were 2.2 ± 0.3, 1.9 ± 0.2, and 1.9 ± 0.2 ng/ml, respectively. Average pretreatment values for cGnRH-II experiments in control, 10, and 100 nM T-treated cells were 1.9 ± 0.2, 1.9 ± 0.3, and 2.1 ± 0.3 ng/ml, respectively. Doses of GnRH giving similar GTH-II values are joined by the same underscore ($p>0.05$; n = 10 columns; Kruskal Wallis test followed by Dunn's test).

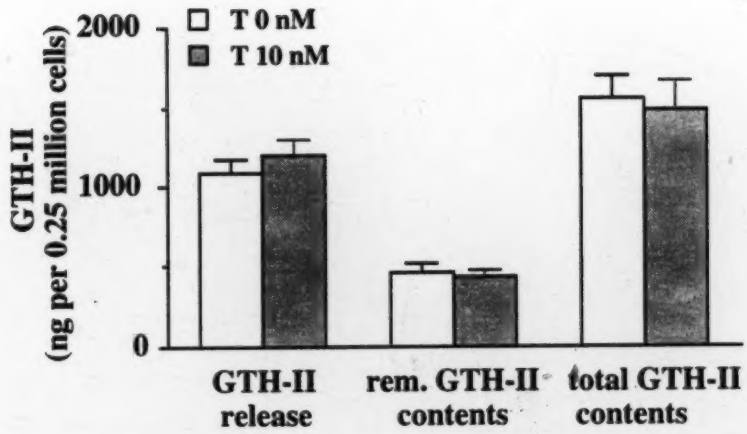

Figure 10. Effects of a 28-h incubation with 10 nM T on the amount of GTH-II released and the remaining cellular GTH-II contents (rem. GTH-II contents) in dispersed pituitary cells from sexually regressed male and female goldfish. Total GTH-II available during the 28-h incubation period was calculated as the sum of the amount released and the amount remaining in the cell. Results presented are mean ± SEM. No significant difference is observed between T-treated and untreated cells ($p>0.05$; $n = 12$; ANOVA followed by Fisher's PLSD test).

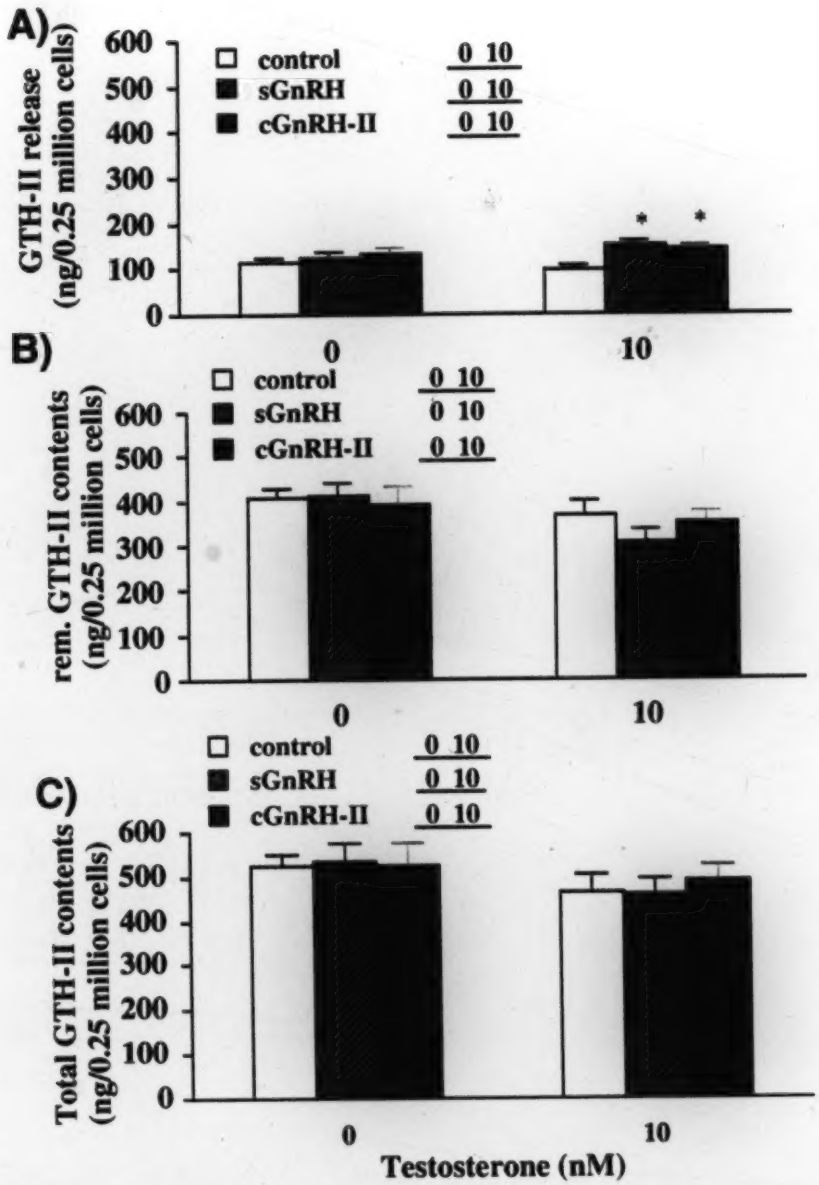

Figure 11. Effects of T on A) GTH-II release, B) remaining (rem.) GTH-II contents, and C) total GTH-II contents following 0.5-h 100 nM GnRH treatment. Dispersed pituitary cells obtained from sexually regressed male and female goldfish were used. Results presented are mean ± SEM. T treatments resulting in similar GTH-II responses are joined by the same underscore ($p>0.05$; $n = 12$; ANOVA followed by Fisher's PLSD test). * denotes significant difference between unstimulated and GnRH-stimulated GTH-II responses ($p<0.05$; ANOVA followed by Fisher's PLSD test).

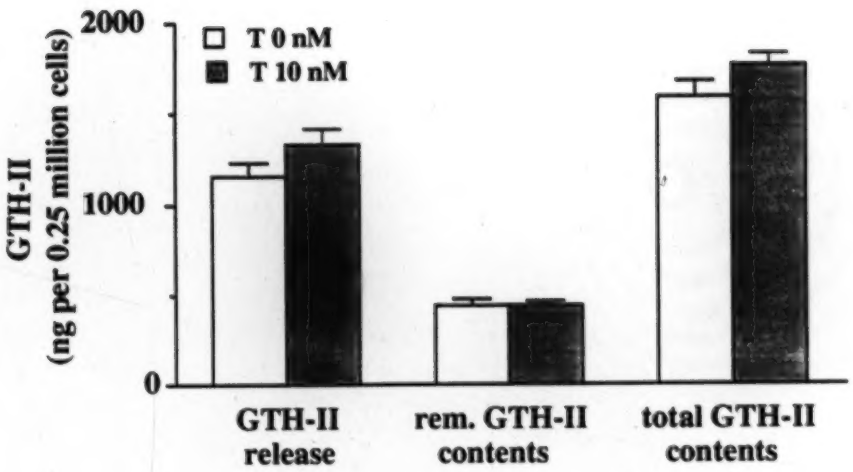

Figure 12. Effects of a 24-h incubation with 10 nM T on the amount of GTH-II released and the remaining cellular GTH-II content (rem. GTH-II contents) in dispersed pituitary cells obtained from sexually recrudescent male and female goldfish. Total GTH-II available during the 24-h incubation period was calculated as the sum of the amount released and the amount remaining in the cell. Results presented are mean ± SEM. No significant difference is observed in the GTH-II values between T-treated and untreated cells ($p>0.05$; $n = 15$; ANOVA followed by Fisher's PLSD test).

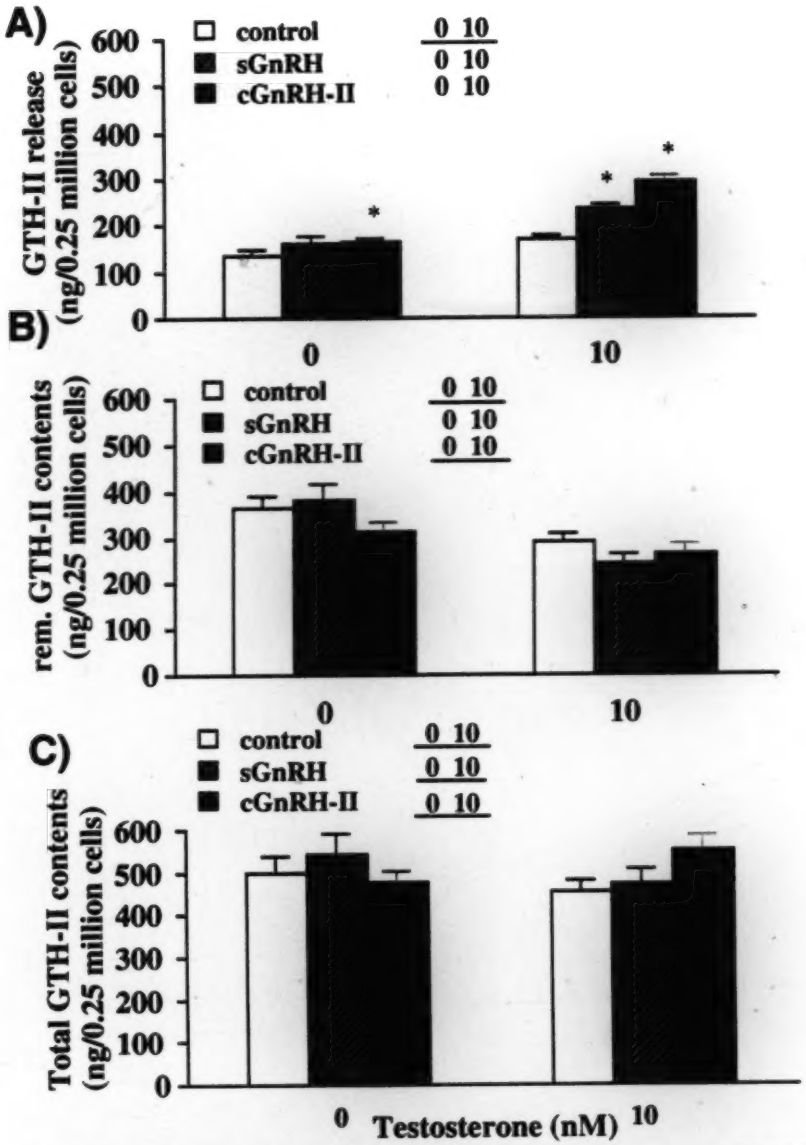

Figure 13. Effects of T on A) GTH-II release, B) remaining (rem.) GTH-II contents, and C) total GTH-II contents following 2-h 100 nM GnRH treatment. Dispersed pituitary cells obtained from sexually recrudescent male and female goldfish were used. Results presented are mean ± SEM. * denotes a significant difference in GTH-II responses between unstimulated and GnRH-stimulated cells ($p<0.05$; $n = 15$; ANOVA followed by Fisher's PLSD test). T treatments resulting in similar GTH-II values are joined by the same underscore ($p>0.05$; ANOVA followed by Fisher's PLSD test).

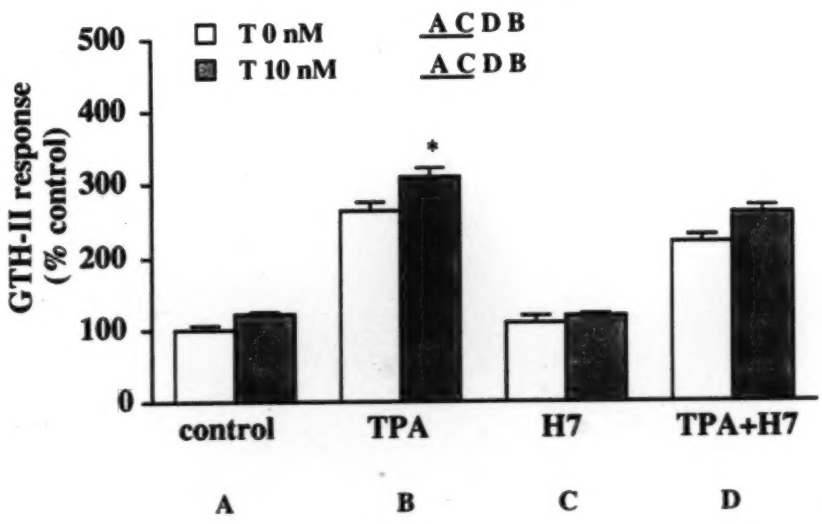

Figure 14. Effects of 10 nM T on 100 nM TPA-induced GTH-II response in the presence or absence of 10 μM H7. Pituitary cells obtained from prespawning male and female goldfish were used. Pooled data from 3 experiments are presented. Treatments are identified by a letter of the alphabet. Results (mean ± SEM) were expressed as a percentage of the values observed in untreated controls. Average basal GTH-II values were 225 ± 23 ng/0.25 million cells. * denotes significant difference between T-treated and untreated groups ($p<0.05$; $n = 9$; Mann-Whitney U test). PKC agonist- or antagonist-treated groups were identified by a letter of the alphabet. Within each T treatment series, groups having similar GTH-II values are marked by the same underscore ($p>0.05$; $n = 9$; Kruskal Wallis test followed by Dunn's test).

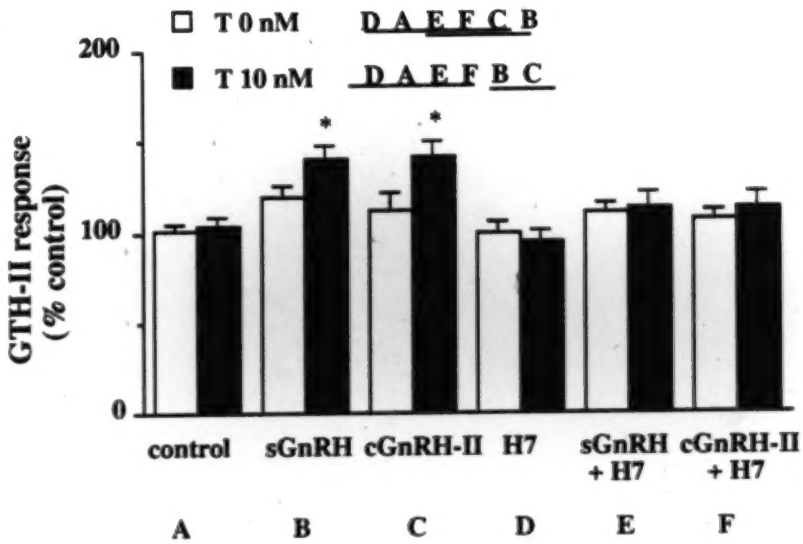

Figure 15. Effects of 10 nM T on 100 nM GnRH-stimulated GTH-II release in the absence or presence of 10 μM H7. Pituitary cells obtained from prespawning male and female goldfish were used. Pooled data from two experiments are presented. Treatments are identified by a letter of the alphabet. Results (mean ± SEM) were expressed as a percentage of the values observed in untreated controls. Average basal GTH-II values were 390 ± 60 ng/0.25 million cells. * denotes significant difference between T-treated and untreated cells ($p<0.05$; $n = 8$; Mann-Whitney U test). Treatment groups are represented by a letter of the alphabet. Within each T treatment series, GTH-II values that are similar are joined by the same underscore ($p>0.05$; $n = 8$; Kruskal Wallis test followed by Dunn's test).

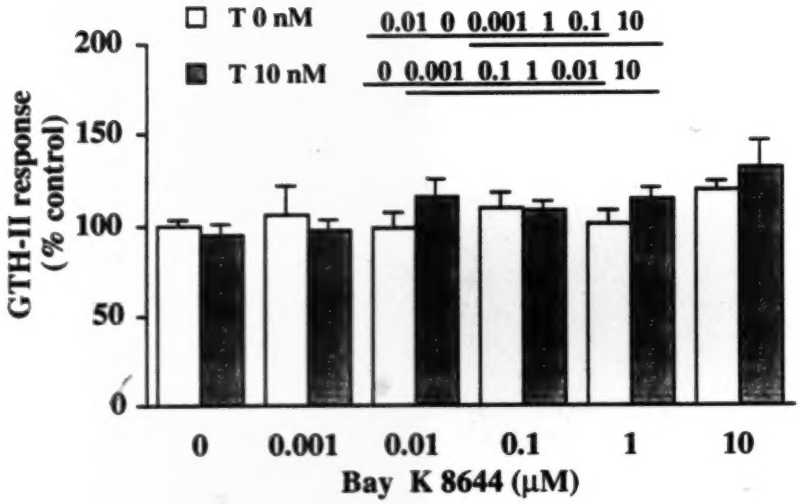

Figure 16. Effects of 10 nM T on the GTH-II response to increasing doses of Bay K 8644. Pituitary cells obtained from sexually regressed male and female goldfish were used. Pooled data from three experiments are presented. Results (mean ± SEM) were expressed as a percentage of the values observed in untreated controls. Average basal GTH-II values were 147 ± 21 ng/0.25 million cells. Within each T treatment series, Bay K 8644-stimulated GTH-II values that are similar share the same underscore ($p>0.05$; n = 8; Kruskal Wallis test followed by Dunn's test). No significant difference is observed between untreated and T-treated groups ($p>0.05$; n = 8; Mann-Whitney U test).

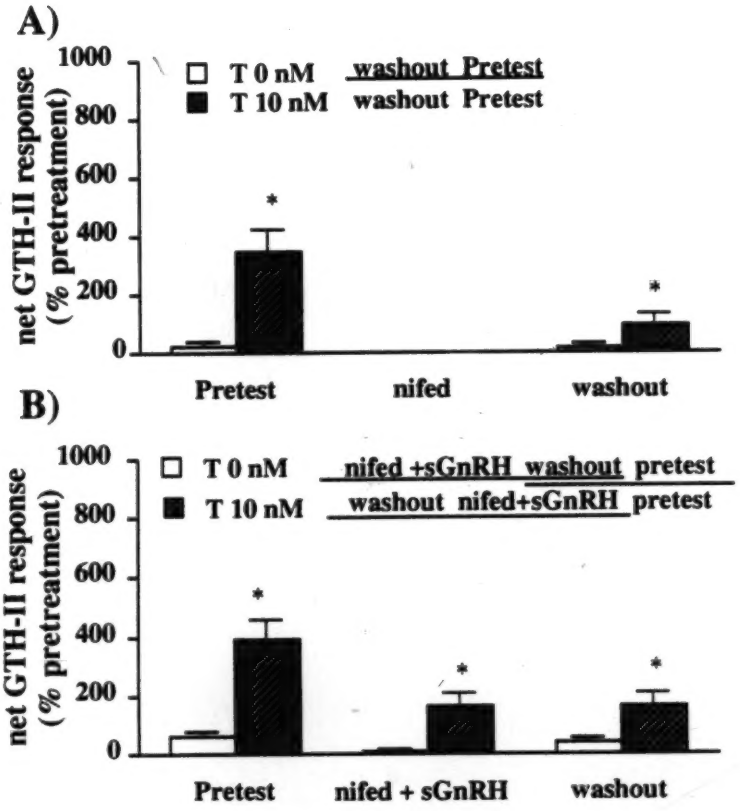

Figure 17. Effects of 10 nM T on 100 nM sGnRH-stimulated GTH-II release in the absence or presence of 1 μM nifedipine (nifed). Dispersed pituitary cells obtained from sexually regressed male and female goldfish were used in cell column perifusion experiments. Net GTH-II responses (mean ± SEM) to 5-min pulse application of sGnRH are presented. A) GTH-II responses to sGnRH pulses applied prior to (pretest) and following (washout) a 70-min application of nifedipine. GnRH was not applied during the nifedipine treatment. Average basal GTH-II values for control and T-treated cells were 2.9 ± 0.4 and 3.0 ± 0.3 ng/ml, respectively (n = 6-8 columns). B) Net GTH-II responses to sGnRH pulses applied before, during, and after nifedipine treatment. Average basal GTH-II values for control and T-treated cells were 2.7 ± 0.3 and 2.2 ± 0.2 ng/ml, respectively (n = 6-8 columns). * denotes significant difference in GTH-II response between untreated and T-treated cells ($p<0.05$; n = 6-8; Mann-Whitney U test). Within each T-treatment series, similar GTH-II values are joined by the same underscore ($p>0.05$; n = 6-8; Kruskal Wallis test followed by Dunn's test).

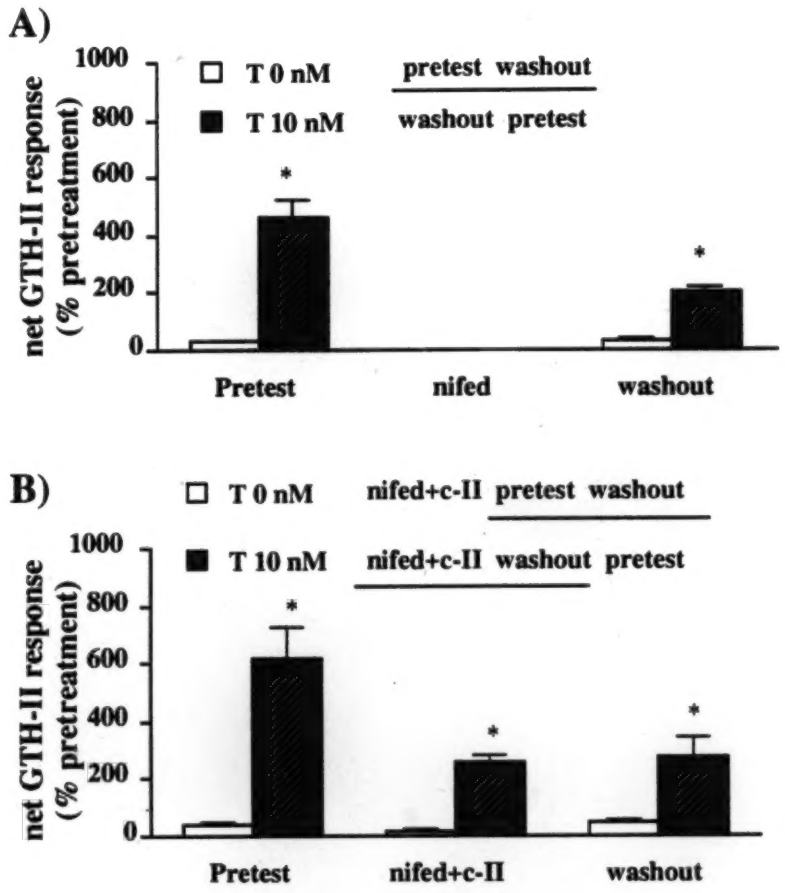

Figure 18. Effects of 10 nM T on 100 nM cGnRH-II-stimulated GTH-II release in the absence or presence of 10 μM nifedipine (nifed) from dispersed pituitary cells obtained from sexually regressed male and female goldfish. A) Net GTH-II responses to two 5-min cGnRH-II pulses separated by a 70-min nifedipine treatment. Averaged basal GTH values for control and T-treated cells were 6.0 ± 0.4 and 6.1 ± 0.4 ng/ml, respectively (mean ± SEM, n = 6-8 columns). B) Net GTH-II responses to three 5-min cGnRH-II pulses in the presence of nifedipine treatment. Averaged basal GTH-II values for control and T-treated cells were 4.9 ± 0.2 and 4.1 ± 0.3 ng/ml, respectively (mean ± SEM, n = 6-8 columns). In each treatment group, significant difference in GTH responses between untreated and T-treated cells is represented by * ($p<0.05$; n = 6-8; Mann-Whitney U test). Within each T treatment series, similar GTH-II values between the different treatment groups are joined by the same underscore ($p>0.05$; n = 6-8; Kruskal Wallis test followed by Dunn's test).

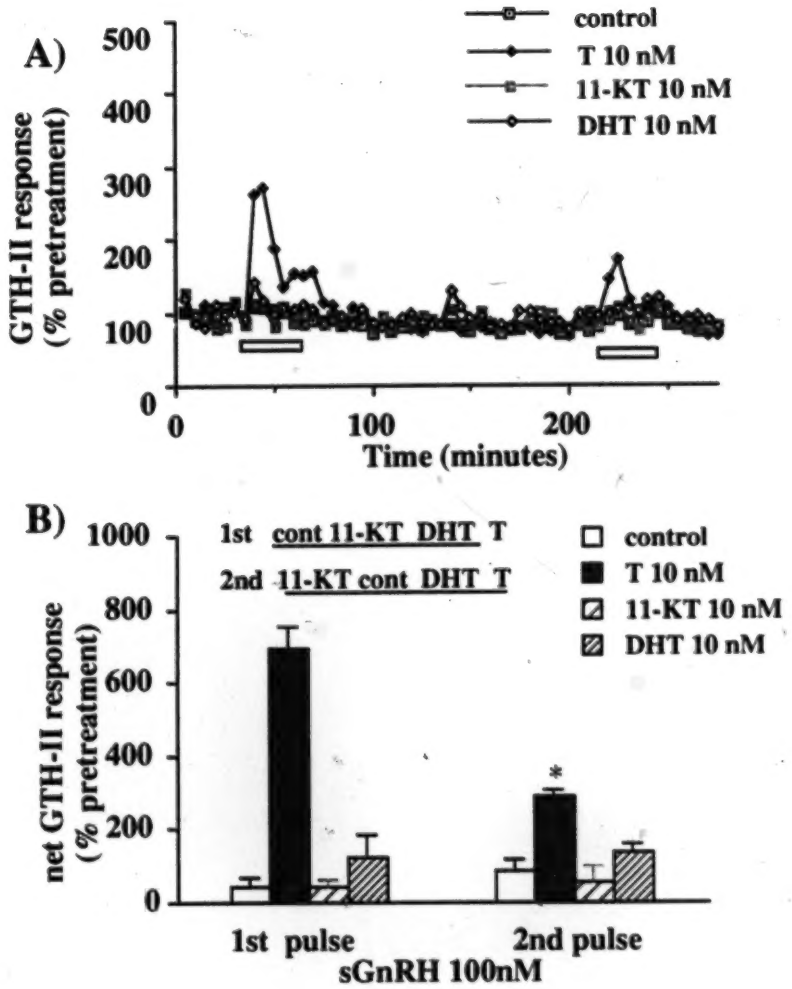

Figure 19. Effects of 10 nM T, 10 nM 11-KT, and 10 nM DHT on the 100 nM sGnRH-stimulated GTH-II response. Dispersed pituitary cells from sexually recrudescent male and female goldfish were used in cell column perifusion experiments. A) Examples of GTH-II release profile are presented. The open bars represent the two 0.5-h sGnRH pulses. B) Net GTH-II response (mean ± SEM) to 100 nM sGnRH calculated from pooled data. Average pretreatment GTH-II values for control, 10 nM T-, 10 nM 11-KT-, and 10 nM DHT-treated columns were similar, being 3.7 ± 0.8, 3.6 ± 0.9, 2.6 ± 0.4, and 3.0 ± 0.9 ng/ml, respectively (n = 4). Androgen treatments resulting in similar GTH-II values share the same underscore ($p>0.05$; n = 4; Kruskal Wallis test followed by Dunn's test). * denotes significant difference between the GTH-II response in the first and second sGnRH pulses ($p<0.05$; n = 4; Wilcoxon signed-rank test).

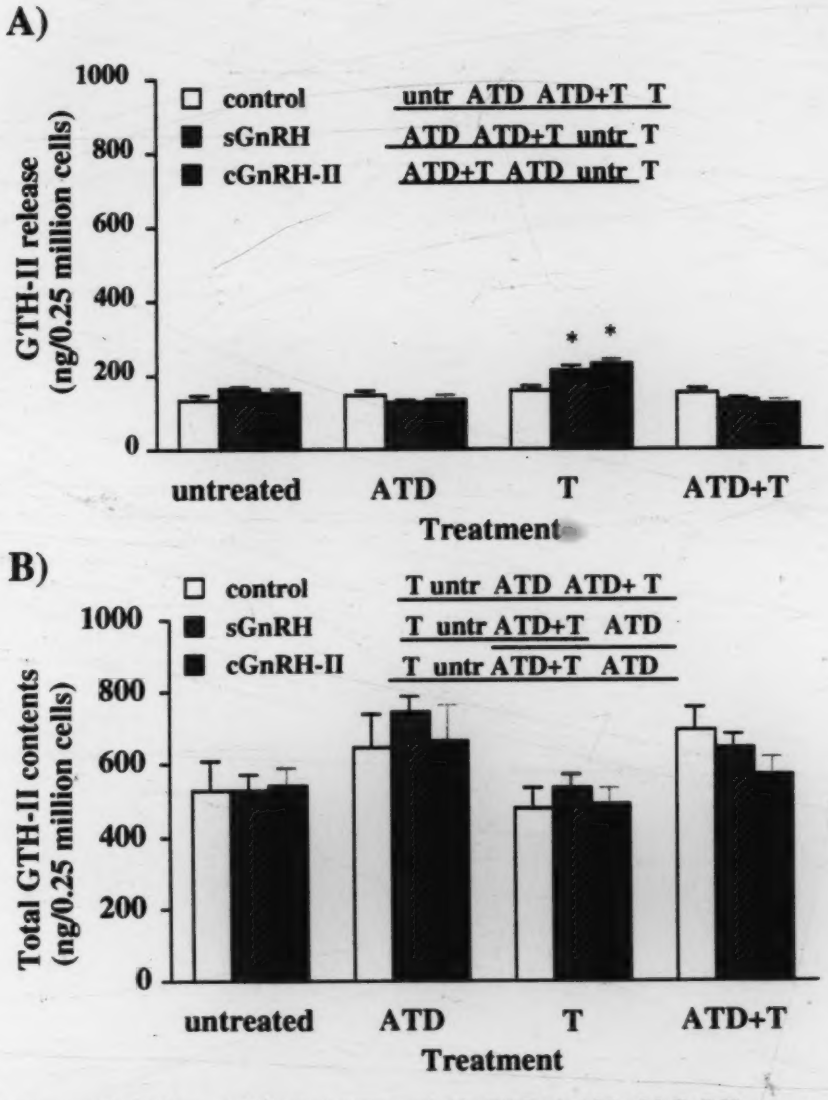

Figure 20. Effects of 300 μM ATD and 10 nM T on 100 nM GnRH-stimulated A) GTH-II release and B) total GTH-II contents from dispersed pituitary cells obtained from prespawning male and female goldfish. GTH-II release that were significantly different between control and GnRH-stimulated groups are marked by * ($p<0.05$; $n = 14$; ANOVA followed by Fisher's PLSD test). In control, sGnRH- and cGnRH-II-treated groups, similar GTH-II values are joined by the same underscore ($p>0.05$; $n = 6$; ANOVA followed by Fisher's PLSD test).

Table III.1. Effects of different doses of T on the net sGnRH-stimulated GTH-II release in (A) the "peak" phase and (B) the "plateau" phase from pituitary cells obtained from sexually regressed, recrudescent, and mature female goldfish.[1]

A)

Gonadal stage	control [2]	T 1 nM	T 10 nM	T 100 nM
regressed	53 ± 22 [a]	115 ± 52 [a]	190 ± 39 *,[a]	272 ± 33 *,[a]
recrudescent	82 ± 20 [a,b]	411 ± 117 *,[b]	485 ± 84 *,[b]	429 ± 43 *,[b]
mature	230 ± 79 [b]	345 ± 44 [b]	751 ± 149 *,[c]	824 ± 234 *,[c]

B)

Gonadal stage	control [2]	T 1 nM	T 10 nM	T 100 nM
regressed	ND [a]	ND [a]	89 ± 53 *,[a]	237 ± 73 *,[a]
recrudescent	10 ± 6 [b]	332 ± 24 *,[b]	256 ± 88 *,[b]	222 ± 57 *,[a]
mature	180 ± 68 [c]	187 ± 16 [c]	330 ± 63 *,[b]	414 ± 122 *,[b]

[1] Values presented are percentages of pretreatment (mean ± SEM).

* denotes significant difference from control values ($p<0.05$; n= 4-8 columns; Dunn's test).

[2] Within each T treatment group, values that are not significantly different from one another are identified by the same superscript letter ($p>0.05$; n= 4-8 columns; Dunn's test).

ND: not detected.

Table III.2. Effects of different doses of T on the net cGnRH-II-stimulated GTH-II release in (A) the "peak" phase and (B) the "plateau" phase from pituitary cells obtained from sexually regressed, recrudescent, and mature female goldfish.[1]

A)

Gonadal stage	control [2]	T 1 nM	T 10 nM	T 100 nM
regressed	27 ± 10 [a]	323 ± 58 [*,a]	298 ± 25 [*,a]	294 ± 41 [*,a]
recrudescent	62 ± 27 [a]	150 ± 44 [b]	459 ± 73 [*,b]	716 ± 83 [*,b]
mature	54 ± 16 [a]	332 ± 33 [*,a]	632 ± 78 [*,b]	727 ± 86 [*,b]

B)

Gonadal stage	control [2]	T 1 nM	T 10 nM	T 100 nM
regressed	3 ± 2 [a]	211 ± 44 [*,a]	218 ± 30 [*,a]	221 ± 45 [*,a]
recrudescent	10 ± 6 [a]	280 ± 37 [*,a]	264 ± 34 [*,a]	321 ± 40 [*,a]
mature	9 ± 5 [a]	160 ± 16 [*,a]	252 ± 59 [*,a]	297 ± 36 [*,a]

[1] Values presented are percentages of pretreatment (mean ± SEM).

* denotes significant difference fom control values ($p<0.05$; n = 6-8 columns; Dunn's test).

[2] Within each T treatment group, values that are not significantly different from one another are identified by the same superscript letter ($p>0.05$; n= 6-8 columns; Dunn's test).

Table III.3. Effects of different doses of T on the net sGnRH- and cGnRH-II-stimulated GTH-II release in (A) the "peak" phase and (B) the "plateau" phase from pituitary cells obtained from prespawning male and female goldfish.[1]

A)

GnRH	control	T 1 nM	T 10 nM	T 100 nM
sGnRH	28 ± 23	381 ± 96	539 ± 44*	572 ± 211*
cGnRH-II	79 ± 24	396 ± 78	565 ± 66*	523 ± 73*

B)

GnRH	control	T 1 nM	T 10 nM	T 100 nM
sGnRH	ND	193 ± 69	242 ± 82*	242 ± 79*
cGnRH-II	54 ± 23	159 ± 117	294 ± 71*	307 ± 36*

[1] Values presented are percentages of pretreatment (mean ± SEM).

* denotes significant difference from control values ($p<0.05$; n= 4 columns; Dunn's test).

ND: non-detected.

Table III.4. Effects of different doses of T on the GTH-II response to 0.01 - 100 nM TPA from dispersed pituitary cells from prespawning male and female goldfish [1].

	Testosterone Treatment (nM)					
	0		1		10	
TPA conc. (nM)	% control [2]	Δ from 0 nM TPA	% control	Δ from 0 nM TPA	% control	Δ from 0 nM TPA
0	100.7 ± 0.8^{a}	0^{a}	$79.4 \pm 5.8^{+,a}$	0^{a}	$76.6 \pm 4.5^{+,a}$	0^{a}
0.01	89.6 ± 4.4^{a}	-11.8 ± 3.4^{a}	84.8 ± 6.1^{a}	8.4 ± 7.6^{a}	80.7 ± 10.4^{a}	3.2 ± 4.8^{a}
0.1	92.2 ± 3.8^{a}	-7.1 ± 4.2^{a}	92.3 ± 9.9^{a}	13.2 ± 4.4^{a}	$95.8 \pm 14.4^{a,b}$	$25.1 \pm 8.7^{+,a,b}$
1	117.0 ± 10.6^{a}	18.3 ± 7.6^{a}	$106.7 \pm 14.2^{a,b}$	$27.0 \pm 8.9^{a,b}$	$129.1 \pm 30.3^{b,c}$	$56.3 \pm 18.9^{+,b,c}$
10	118.1 ± 5.6^{b}	20.6 ± 5.6^{b}	119.5 ± 14.6^{b}	37.5 ± 9.8^{b}	$131.5 \pm 25.1^{b,c}$	$64.1 \pm 11.8^{+,b,c}$
100	122.4 ± 6.1^{b}	25.8 ± 6.9^{b}	$99.5 \pm 8.0^{a,b}$	$21.1 \pm 6.9^{a,b}$	135.8 ± 16.8^{c}	$68.3 \pm 10.5^{+,c}$

[1] Pooled data from 4-5 experiments are presented (n = 12-15). Results (mean ± SEM) were expressed as a percentage of the value observed in untreated controls. Average basal GTH-II values were 312 ± 45 ng/0.25 million cells.

[2] GTH-II values with different superscripted letters are statistically different ($p<0.05$; Dunn's test).

[+] denotes significant difference from the 0 nM T-treated group ($p<0.05$; Dunn's test).

Table III.5. Effects of 10 nM T on the GTH-II response induced by 0.1 - 100 μM ionomycin in dispersed pituitary cells from postspawning male and female goldfish[1].

	Testosterone Treatment (nM)			
	0		10	
Ionomycin conc. (μM)	% control [2]	Δ from 0 μM Ionomycin	% control	Δ from 0 μM Ionomycin
0	100.0 ± 0.1 [a]	0 [a]	67.5 ± 11.3+,[a]	0 [a]
0.1	102.0 ± 7.1 [a]	3.8 ± 6.3 [a]	80.8 ± 13.5 [a]	13.9 ± 4.3 [a]
1	113.1 ± 7.2 [a,b]	14.6 ± 5.9 [a]	106.0 ± 19.4 [a,b]	40.8 ± 6.3+,[a,b]
5	180.9 ± 17.4 [b,c]	83.0 ± 11.7 [b]	156.1 ± 32.0 [b,c]	79.7 ± 11.2 [b,c]
10	177.7 ± 30.3 [c]	86.1 ± 16.0 [b]	164.8 ± 38.3 [b,c]	105.6 ± 20.1 [b,c]
100	260.6± 9.7 [c]	161.1 ± 13.2 [b]	262.0 ± 72.3 [c]	199.7 ± 42.7 [c]

[1] Pooled data from 4-5 experiments are presented (n = 9). Results (mean ± SEM) were expressed as a percentage of the value observed in untreated controls. Average basal GTH-II values were 217 ± 19 ng/0.25 million cells.

[2] GTH-II values with different supercripted letters are statistically different ($p<0.05$; Dunn's test).

+ denotes significant difference from the untreated group ($p<0.05$; Mann-Whitney U test).

IV. *Discussion*

IV.1. Seasonality of basal GTH-II secretion and responsiveness to GnRH in non T-treated cells.

In goldfish, plasma GTH-II level increases with gonadal maturity (Kagawa *et al*, 1983; Habibi *et al*, 1989a). In the present study, seasonal differences in the basal GTH-II release can also be demonstrated in primary culture of dispersed pituitary cells obtained from goldfish of different gonadal maturity. Basal GTH-II release from pituitary cells maintained under normal static incubation conditions increased with advancing gonadal maturity (147 ± 21 ng/ml, regressed, Fig. 16; 225 ± 23 ng/ml, recrudescing, Fig. 14; 390 ± 60 ng/ml, prespawning, Fig. 15). These observations suggest that seasonal reproductive influences on basal GTH-II release present *in vivo*are still retained in the pituitary cells, even after the removal of pituitaries from the goldfish. In contrast to static incubation studies, the basal GTH-II levels from perifused pituitary cells did not show prominent seasonal differences (Figs. 1-9, 17-19). The difference in the seasonality of basal release between static incubation and perifusion experiments could be due to the difference in the experimental protocol. The GTH-II release measured in perifusion experiments was accumulated over 5 min, whereas those in static culture were accumulated over a 2-h period. Thus, seasonal differences in basal GTH-II values may become more prominent in static incubation experiments than in perifusion studies.

Previous studies indicate that the GTH-II secretion response to *in vivo* injections of GnRH or GnRH analogs increases in magnitude with gonadal maturation of goldfish. This increase in responsiveness has been partly attributed to an increase in GnRH receptor numbers in the pituitary (Habibi and Peter, 1991b). Results from the present study indicate that pituitary cells obtained from female goldfish at later stages of

ovarian recrudescence also showed greater responses to a 0.5-h sGnRH pulse than cells prepared from fish at earlier stages of ovarian maturation. (Net GTH-II response to first 100 nM sGnRH pulse in non-T-treated controls: regressed, 52 ± 23, n = 4-6; recrudescing, 87 ± 21, n = 6-8; prespawning, 366 ± 126 % pretreatment, n = 6-8, $p<0.05$, prespawning vs. earlier stages of ovarian maturation, Dunn's test; Figs. 1-3). These observations suggest that increasing sexual maturity affects the sGnRH-induced GTH-II release from goldfish pituitary cells.

In addition to the influence on the total GTH-II response, seasonal reproductive conditions affected the hormone release profile of the *in vitro* GTH-II response to sGnRH. When the "peak" and "plateau" phases of GTH-II response to sGnRH were analyzed, it is apparent that the magnitude of the "peak" response to sGnRH increased progressively from experiments with pituitary cells from sexually regressed animals to experiments with cells from sexually mature female goldfish. The "plateau" phase of the GTH-II response to sGnRH also increased with gonadal maturity. Thus, with ovarian recrudescence, the magnitude and duration of the *in vitro* GTH-II response to sGnRH increased, while the release profile changed from a "monophasic" to a "biphasic" nature. The presence of a "biphasic" response to sGnRH from pituitary cells prepared from fish in later stages of gonadal recrudescence, but not from cells obtained from sexually regressed fish, has been proposed by Jobin *et al* (1996a). In this earlier study, a 10-min sGnRH pulse challenge protocol was used and a clear distinction between "peak" and "plateau" phase could not be consistently identified. The acute GTH-II response to sGnRH was reported to approach a "biphasic" nature only in experiments with cells prepared from fish at later stages of recrudescence. The present experiments represent a much clearer demonstration for the existence of a seasonal influence on the kinetics or "phasic" nature of the response to sGnRH.

In contrast to the responses to sGnRH, the GTH-II response to cGnRH-II were generally consistent throughout the seasonal reproductive cycle. The net GTH-II responses to a 0.5-h cGnRH-II pulse from cells prepared from female goldfish were comparable among the three stages of gonadal maturation examined. (Net GTH-II response to first 100 nM cGnRH-II pulse in non-T-treated controls: regressed, 30.5 ± 11.5, n = 6; recrudescence: 75.1 ± 34.1, n = 6-8; matured: 62.5 ± 17.5 % pretreatment, n = 6; $p>0.05$ vs one another, Kruskal Wallis test; Figs. 4-6). These observations suggest that the status of the ovarian recrudescence has no great influence on the magnitude, the duration, and the "biphasic" nature of the GTH-II responses to cGnRH-II.

The reasons for these observed differences between the seasonal reproductive effects on the kinetics profile of the GTH-II response to sGnRH and cGnRH-II are not known. In rat gonadotropes, the initial "peak" phase of the biphasic LH response to GnRH depends mostly on the mobilization of calcium from intracellular stores, whereas the sustained "plateau" phase is largely dependent upon the availability of extracellular calcium (Catt and Stojilkovic, 1989). In goldfish, cGnRH-II and sGnRH stimulation of GTH-II release differ in terms of their relative dependence on extracellular and intracellular Ca^{2+} mobilization, and arachidonic acid metabolism (see section I.3). Seasonal differences in the kinetic profile of the GTH-II response to the two native GnRHs may reflect these dissimilarities in their signal transduction mechanisms and the differential effects of gonadal/seasonal influences on these signalling pathways. This hypothesis requires further testing.

Comparing the seasonal reproductive influences on the effectiveness of the two native GnRHs in stimulating GTH-II release from dispersed goldfish pituitary cells, several other observations can be made. In cells prepared from regressed female goldfish, cGnRH-II could induce a GTH-II response of longer duration than sGnRH. This indicates that the response to prolonged exposure of cGnRH-II is less easily

"desensitized" at this stage of gonadal development. In contrast, in later stages of ovarian maturation, the size of both the "peak" and the "plateau" response to cGnRH-II are smaller than those induced by sGnRH, while the duration of the GTH-II responses to both GnRHs are similar. It is possible that sGnRH and cGnRH-II have different physiological roles in regulating ovarian maturation in goldfish. As the goldfish grows from a prepubertal stage into an adult, the increase in pituitary sGnRH contents is greater than the increase in cGnRH-II (Rosenblum *et al*, 1994). In pituitary fragments of adult goldfish, the *in vitro* secretion of sGnRH in response to K^+ is also higher than that of cGnRH-II (Rosenblum *et al*, 1994). Taken together with results from the present study, these observations suggest that although cGnRH-II is equally effective in stimulating GTH-II release throughout the seasonal reproductive cycle, it may be more important than sGnRH in regulating GTH-II secretion during initial pubertal development and initial stages of ovarian development. In contrast, sGnRH may have a more important role than cGnRH-II in regulating pituitary GTH-II release in later stages of ovarian maturation. Whether this hypothesis is true or not requires further investigation.

IV.2. Effects of T on GnRH-stimulated GTH-II release in the seasonal reproductive cycle.

IV.2.1. *Presence of a direct positive effect of T on GnRH-induced GTH-II throughout the seasonal reproductive cycle.*

Previously, it has been demonstrated that T acts at the level of the goldfish pituitary to potentiate GTH-II response to LHRH-A (Trudeau *et al*, 1991; Trudeau *et al*, 1993a). However, whether T has any direct effect on goldfish pituitary cells to stimulate GTH-II release is not known. This study is the first to demonstrate

that T directly exerts a positive effect on GnRH-stimulated GTH-II release at the level of goldfish pituitary cells. Overnight pre-incubation with T potentiated the sGnRH and cGnRH-II-induced GTH-II release from pituitary cells obtained from female goldfish at different sexual stages in perifusion studies (Figs. 1-6). Similarly, T pretreatment enhanced the GTH-II response to the two native GnRHs in perifusion experiments with cells prepared from both male and female goldfish at three stages of gonadal recrudescence (sexually regressed, Figs. 9, 17 and 18; recrudescing, Figs. 19; prespawning, Fig. 7 and 8). Likewise, the sGnRH- and cGnRH-II-elicited GTH-II release in static incubation studies were greater in T-treated than in non-treated pituitary cells from both male and female at either sexually regressed (Fig. 11), recrudescing (Fig. 13), or prespawning stages (Fig. 20). These observations clearly demonstrate that T exerts a direct positive influence on GnRH-stimulated GTH-II release in goldfish; furthermore, this effect of T is present in female goldfish at all stages of the seasonal reproductive cycle. In the present study, T was effective in enhancing the GTH-II release to both 5-min and 0.5-h exposures to GnRH in perifusion experiments, as well as to 0.5- and 2-h GnRH challenges in static incubation experiments. Thus, the potentiating effect of T extends to both short- and long-term GnRH actions on GTH-II release.

In the initial experiments of this study, the effects of 1 to 100 nM T on the GnRH-stimulated GTH-II responses were tested. Examining the GTH-II response to the first GnRH pulse, the potentiating effects of T were dose-dependent in experiments with cells obtained from sexually regressed and sexually mature females for sGnRH studies (Figs. 1 and 3), and in sexually recrudescing and sexually mature females for cGnRH-II studies (Figs. 5 and 6). Similarly, for the GTH-II response to the second GnRH pulse challenge, differences in the effectiveness of different doses of T in increasing the GTH-II responses were observed in experiments with cells prepared from sexually recrudescing females for both native GnRH forms (Figs. 2 and 5), and

in cells from sexually mature females for cGnRH-II (Fig. 6). Generally speaking, these results indicate that the ability of T to enhance GnRH-stimulated GTH-II secretion is not a "threshold" effect, but is a dose-dependent action. It is also apparent that 10 nM T is generally the lowest dose that can exert an enhancing influence on GnRH-induced GTH-II responses in pituitary cells obtained from either females alone, or from both males and females, and irrespective of gonadal maturational stages (Figs. 2-8). Thus, 10 nM T is often the only dose of T used for subsequent experiments in this thesis.

Despite the presence of a potentiating effect on GnRH-stimulated GTH-II release, preincubation with T did not affect basal GTH-II secretion in all perifusion experiments in this thesis. T also had no effects on basal GTH-II release in all but two series of static incubation experiments. This is in agreement with previous studies by Trudeau *et al* (1991, 1993a) which indicated that T implantations also did not alter basal GTH-II release from goldfish pituitary fragments. Therefore, T generally has no effects on basal GTH-II release. The selective enhancement of T on stimulated secretion, as opposed to basal release, further indicates that these two release processes are mediated or regulated by dissimilar mechanisms. It is well known that exocytosis in most cells is separated into a constitutive and a regulated pathway (Alberts *et al*, 1994). It is likely that the direct effects of T on exocytotic activity in gonadotropes is restricted to elements in the regulated pathway or those components modulating it.

IV.2.2. *Effects of T on GnRH-induced desensitization of the GTH-II response.*

In perifusion experiments presented in this thesis, repeated GnRH challenges were used to assess the possible influence of T on GnRH-induced desensitization of GTH-II release. The two native GnRHs were applied either as 30-min pulses at 2.5-h intervals(Figs. 1-8, 19) or as 5-min pulses at 1-h intervals (Figs.

17-18). In both test protocols, the GTH-II response to GnRH in untreated cells showed no desensitization regardless of the gonadal conditions of the goldfish used. In contrast, although T-pretreatment enhanced the GTH-II responses to the first pulse-application of GnRH, the second GnRH-stimulated GTH-II responses in T-treated cells were, in general, desensitized when compared to the response observed in the first GnRH pulse in all experiments with pituitary cells from female goldfish using 0.5-h GnRH pulse challenges (Figs. 2-6). Similarly, desensitization to the second 0.5-h exposure of GnRH was also observed in T-treated cells derived from both male and female godlfish (Figs. 7, 8 and 19). Some components important for the potentiating effect of T might not have been replenished during the rest period between the two, relatively long, 0.5-h GnRH pulses. Although the identity of this component(s) is as yet unknown, a hypothesis on the physiological significance of this phenomenon of desensitization following T pretreatment can be made. During later stages of ovarian recrudescence, circulating T levels in goldfish generally increase (Kagawa *et al*, 1983). When the negative neuroendocrine influence on GTH-II release is removed, such as during the removal of DA inhibition in the periovulatory period (Sloley *et al*, 1991), the full positive feedback influence of T will be transiently expressed. The stimulatory action of T also increases the ease of GnRH-induced desensitization. This latter effect limits the duration of the elevated GTH-II response in the pituitary and could be one of the mechanisms leading to the termination of the periovulatory GTH-II surge in females.

IV.2.3. *Effects of ovarian conditions on the magnitude and kinetic profile of the T-potentiated GTH-II response to GnRH.*

In T-treated cells, the net GTH-II response to the first pulse application of sGnRH and cGnRH-II increased with increasing ovarian maturity (Figs. 1-6). For

example, in cells treated with 10 nM T, the net GTH-II response to sGnRH increased from 214 ± 69% pretreatment during gonadal regression to 651 ± 152% during times of gonadal recrudescence; by prespawning, the net GTH-II response to sGnRH was 1066 ± 187% ($p<0.05$ vs each other; Dunn's test). Likewise, the response to cGnRH-II in experiments done at times of gonadal regression (480 ± 58% pretreatment) were significantly less than corresponding responses in experiments performed at times of gonadal recrudescence and prespawning (728 ± 92 and 891 ± 122% pretreatment, respectively; $p<0.05$ vs regressed, Dunn's test). Similar trends were observed in 1 and 100 nM T-treated groups. These results suggest that the effectiveness of T in potentiating the GTH-II responses to the two native GnRHs increases with advances in gonadal maturity.

The GnRH-stimulated GTH-II release in T-treated cells was also separated into "peak" and "plateau" phase responses in order to evaluate the influence of T, as well as that of the ovarian maturational conditions, on the phasic nature of the hormone response. T treatment in general, and 10 and 100 nM T in particular, increased the magnitude of both the "peak" and "plateau" responses to either sGnRH or cGnRH-II relative to untreated cells irrespective of the gonadal conditions of the pituitary donors (Figs. 1-6, Tables III.1 and III.2). This indicates that both components of the "biphasic" GTH-II response contribute to the enhanced stimulatory effects of GnRH following T pretreatment at all stages of ovarian maturation.

Although both "peak" and "plateau" response phases contributed to the enhanced GnRH-stimulated secretion following T treatment in all ovarian maturation stages, the effectiveness of T to influence the magnitude of these two different phases of the GnRH-induced GTH-II release differed according to ovarian conditions of the pituitary donor. For example, in 10 nM T-treated cells, the magnitude of the "peak" and "plateau" responses to sGnRH were smaller in experiments with cells from sexually regressed fish than in experiments with pituitary cells obtained from animals at later

stages of ovarian recrudescence (Table III.1). Similar trend also existed for 100 nM T-treated cells (Table III.1). These data suggest that the ability of T to enhance the "peak", as well as the "plateau" response, to sGnRH increases with ovarian maturation.

The seasonal influence on the ability of T to alter the two GTH-II release phases in response to cGnRH-II appears to be different from that for sGnRH. In 10 and 100 nM T-treated cells, the magnitude of the "peak" responses to cGnRH-II were greater in experiments with cells obtained from sexually mature fish than from regressed stages (Table III.2A). However, a similar trend was not observed with the 1 nM T-treated cells. The "plateau" response to cGnRH-II in all T-treated cells were also not different between the three sexual recrudescence stages (Table III.2B). Thus, unlike the situation for sGnRH, increasing ovarian maturation elevated only the ability of the two higher doses of T to enhance the "peak", but not the "plateau" response, to cGnRH-II stimulation.

IV.2.4. *Differences between the GTH-II releasing actions of the two native GnRHs in response to T treatment.*

Although T is effective in potentiating GTH-II release to the two native GnRHs, differences between the effects of T on sGnRH- and cGnRH-II-induced responses are observed (Tables III.1 and III.2). First, cGnRH-II-induced GTH-II release is more sensitive to T action than sGnRH-induced release especially at early stages of ovarian development. One nM T effectively potentiated the GTH-II responses to cGnRH-II, but not to sGnRH, in sexually regressed female goldish pituitary cells (Figs. 1 and 4). In contrast, the effectiveness of different doses of T on sGnRH- and cGnRH-II-stimulated GTH-II were generally similar for experiments done at later stages of gonadal maturation (Figs. 3, 6-8). Second, as discussed in section IV.2.3, gonadal maturation affects the potentiating effects of T on both phases of the "biphasic"

GTH-II response to sGnRH. But gonadal maturation affected only the "peak" phase of the cGnRH-II-induced GTH-II response in T-treated cells. The reasons for these differences in the sGnRH and cGnRH-II actions in response to T treatment is not known. Since the two GnRHs utilize dissimilar signal transduction mechanisms in stimulating goldfish GTH-II release (reviewed in Chang *et al*, 1996 and section I.3), it is likely that T may be acting differently on their respective intracellular pathways (also see discussion on T action on GnRH signal transduction pathway below, section IV.3).

IV.2.5. *Effects of gonadal sex of the donor goldfish on the potentiating actions of T on GnRH-stimulated GTH-II release from pituitary cells.*

The effectiveness of T to potentiate the GnRH-induced GTH-II release in pituitary cells from male and female goldfish was comparable to those seen in cells from female goldfish. These results imply that T is as effective in potentiating GTH-II responses to GnRH stimulation in pituitary cells from both male and female goldfish, as in cells from females alone. Since the presence of cells from male pituitary donor did not affect the positive actions of T on GnRH-stimulated GTH-II release, pituitaries from both sexes of goldfish were used in subsequent experiments to examine the mechanisms by which T treatment enhances GnRH-induced GTH-II secretion. But whether these effects of T on GTH-II release are present in cells from male pituitary donor alone require further investigation.

IV.3. Mechanisms by which T potentiates GnRH-induced GTH-II release.

IV.3.1. *Effects of T on GnRH receptor binding.*

One possible way by which T exerts its potentiating actions on GnRH-stimulated GTH-II release from goldfish pituitary cells is by increasing GnRH receptor binding affinity and/or capacity. However, this possibility is unlikely. *In vivo* implantation of T did not affect sGnRH binding affinity or capacity in pituitary membrane preparations in an earlier study (Trudeau *et al*, 1993a). Preliminary studies in this thesis indicate that pretreatment of goldfish pituitary cells with 10 nM T for 24 h did not increase cGnRH-II receptor binding capacity (Appendix 2). In fact, the maximal cGnRH-II binding in dispersed goldfish pituitary cells were consistently lower in T-pretreated cells than in non-treated cells in these preliminary trials. T pretreatment resulted in a decrease in specific binding capacity of GnRH receptor to approximately 14 % of that in untreated cells (Appendix 2). Furthermore, the estimated potencies of the GTH-II dose-response curves to both GnRHs tended to shift to the right following treatment with T (Fig. 9, section III.2). These results suggest that T-induced increase in GTH-II release to GnRH stimulation is not a consequence of an increase in GnRH receptor number or receptor binding affinity.

IV.3.2. *Effects of T on GTH-II synthesis.*

Another possible mechanism by which T potentiates stimulated GTH-II response is by elevating the amount of GTH-II available for release in gonadotropes as a result of T action on GTH-II synthesis. Previous studies in teleosts (goldfish, Huggard *et al*, 1996, see section I.2.5.3; tilapia, Rosenfeld *et al*, 1996, Melamed *et al*, 1996; black carp, Gur *et al*, 1995, Yaron *et al*, 1995; African catfish, Rebers *et al*, 1997, see section I.6), as well as in mammals (rats, see section I.7), indicate that steroids, including T, can increase gonadotropin subunit mRNA synthesis and *de novo* GTH protein production. In the present study, dispersed pituitary cells were incubated with T for either 28 or 24 h before the commencement of GnRH treatments. During this

period of incubation, T did not alter the total GTH-II contents or the amount of GTH-II remaining in the cells (Figs. 10 and 12). Thus, overnight T treatments did not increase GTH-II peptide production and the amount of GTH-II in cells available for GnRH stimulation of release.

T treatments were ineffective in increasing the GTH-II contents before the GnRH challenge within the pituitary cells; however, the potentiated GTH-II release in response to GnRH could result from a positive modulation of GnRH-induced synthesis by T. In goldfish, as well as in other teleosts, GnRH has been shown to increase GTH-II β-subunit mRNA levels in the pituitary (Khakoo *et al*, 1994). In the present study, neither a 0.5- or 2-h treatment with GnRH increased total pituitary GTH-II contents in T-treated or untreated cells (Figs. 11 and 13). These observations suggest that *in vitro* applications of T did not increase the GTH-II release response by increasing GTH-II peptide synthesis in goldfish pituitary cells during GnRH stimulation. These results also indicate that steroidal control of GTH-II release and synthesis in the goldfish are likely to be dissociated. Evidence from the mammalian literature suggests that this is highly likely in rats. Pretreatment of rat anterior pituitary cells with E_2 increases the sensitivity of the gonadotropes to GnRH stimulation of LH biosynthesis (Ramey *et al*, 1987). The major effect of E_2 is to lower the effective physiological concentration of GnRH needed to stimulate LH glycosylation and LH release. But the enhancement in GnRH-stimulated LH synthesis caused by E_2 treatment cannot fully account for its ability to increase GnRH-stimulated LH release. Thus, it appears that E_2 has separate effects on the mechanisms regulating GnRH-stimulated LH release and LH biosynthesis in rat pituitary cells.

In the present study, the lack of an effect of T on increases in total GTH-II contents, both prior to and during GnRH treatment, seems to contradict other results in the goldfish literature (Huggard *et al* , 1996; see section I.2.5.3). There could be at least five possible explanations for this discrepancy.

First, differences between the present study with dispersed pituitary cells, and previous *in vivo* experiments and *in vitro* studies with pituitary fragments, could be due to the differences in direct vs. indirect T action. T applied *in vivo* could have exerted its effects through modulation of the release of hypothalamic factors. The use of pituitary fragments also does not remove possible T effects on the secretion of neuroendocrine factors from remaining nerve terminals and their subsequent actions. However, such indirect actions of T would not have been possible in the present study with dispersed pituitary cells.

Second, there is a difference in the length of incubation with T between the present, and the previous *in vivo* and *in vitro* studies (Huggard *et al*, 1996). A longer incubation time than that used in this thesis might have been required for T to promote GTH-II synthesis since *in vivo* application of T for 72 and 96 h were effective in increasing GTH-II subunit mRNA synthesis (Huggard *et al*, 1996). However, a recent *in vitro* study on goldfish pituitary fragments suggested that a 15-h exposure to T was sufficient to elevate GTH-II subunit mRNA levels (Huggard *et al* , 1996). Whether the duration of T treatment is the correct explanation for the lack of T influence on pituitary cellular GTH-II contents is unclear.

Third, the doses of T used in previous studies were likely superphysiological. This is true even for the closest comparable *in vitro* study by Huggard *et al* (1996) with goldfish pituitary fragments, in which the effective doses of T for stimulating GTH-II subunit mRNA production from pituitary fragments obtained from goldfish in sexually immature stages *in vitro* were 2 to 5000 ng/ml of T (i.e., 7 nM to 20 μM). However, 200 nM and lower concentrations of T were without effect on pituitary fragments from sexually mature fish. Physiologically, approximately 0.5 and 28 ng/ml of (≈ equivalent to 2 and 100 nM) T are found in the circulation of sexually regressed and mature goldfish, respectively (Huggard *et al*, 1996). The higher doses of T used in the study by Huggard *et al* (1996) are likely superphysiological and

thus cannot be an accurate representation of the physiological situation. On the other hand, the concentration of T used in the present study is 10 nM, approximately equivalent to 2.9 ng/ml, and is within the physiological concentration of T in circulation. It is possible that T at physiological concentrations does not affect GTH-II peptide synthesis *in vitro*, especially when applied only for a relatively short duration.

Fourth, an increase in GTH-II subunit mRNA production does not necessarily represent a simultaneous increase of GTH-II protein contents in the goldfish pituitary. In mammals, the mechanisms regulating LH biosynthesis are complex and involve multiple steps. In the rat, the biosynthetic pathway for LH and other glycoprotein hormones is thought to involve transcription and translation of separate mRNAs for the α- and β-subunits, as well as several cotranslational and posttranslational glycosylation steps, before the final LH polypeptide is produced (Hussa, 1980; Chappel *et al*, 1983). It is likely that the biosynthetic pathway of GTH-II also involves multiple steps. Thus, an increase in GTH-II mRNA levels might not represent a simultaneous increase in GTH-II protein contents.

Fifth, differences between the steroid milieu in the present and previous experiments may also be responsible. T, whether applied *in vivo* or *in vitro*, can be aromatized or converted to other metabolites (Trudeau *et al*, 1991b; Appendix 1). Thus, T can act alone or as one of its many different metabolites. In rats, evidence is available to suggest that T and its metabolites may have different actions on the hormone synthesis pathway. Yasin *et al* (1996) reported that DHT enhances LH β-subunit mRNA production whereas T stabilizes LH β-subunit mRNA levels. The effects of E_2 in rats is mainly to potentiate GnRH actions on gonadotropin synthesis by increasing the sensitivity of the gonadotropes to GnRH (Ramey *et al*, 1987). Similarly, a balance of T and its metabolites may also be important in determining the effects of T in teleosts such as the goldfish. In the current experiments, the presence of an aromatase inhibitor, ATD, significantly increased the GTH-II contents under the stimulation of sGnRH

(Fig. 20). Total GTH-II contents also tended to be higher in ATD-treated than in non-ATD-treated groups. In view of the observations that exposure to high levels of T decreased GTH-II β-subunit mRNA levels and blocked the ability of GnRH to stimulate an increase in GTH-II subunit mRNA concentrations in goldfish (Habibi *et al*, 1997), the present data suggest that an aromatized T metabolite (perhaps E_2) may have an attenuating influence on the GTH-II synthesis process. Although it has not been studied in goldfish, the promoter region in the GTH-II β-subunit gene in rainbow trout is known to contain several estrogen responsive elements (Le Drean *et al*, 1997). Thus, it is possible that E_2 may directly regulate GTH-II subunit mRNA synthesis in teleosts. After the blockade of aromatase action by ATD, the inhibitory influence of aromatized androgen metabolites is diminished and the effects of nonaromatizable androgens likely become more prominent. Nonaromatizable androgens have been shown to positively modulate processes leading to GTH-II biosynthesis in teleosts. Huggard *et al* (1996) showed that *in vivo* treatment with 11β-hydroxyandrosterone (20 μg/fish), a nonaromatizable androgen, increased GTH-II subunit mRNA production in goldfish pituitary. Similarly, Rebers *et al* (1997) demonstrated that 11-KT increased GTH-II subunit mRNA levels in cultured pituitary cells of the African catfish. Taken together, these results further suggest that nonaromatizable and aromatizable androgens have different physiological actions on GTH-II biosynthesis and secretion in goldfish. Therefore, a balance of gonadal steroids with their different metabolites likely determines the gonadotrope responses in the goldfish pituitary. Changes in the gonadal steroid milieu is an important factor in the regulation of circulating GTH-II levels throughout the seasonal reproductive cycle.

IV.3.3. *Effects of T on GnRH post-receptor signal transduction pathways leading to GTH-II release.*

Since T did not change cellular contents of GTH-II or increase GnRH binding, it is possible that T potentiates GnRH-stimulated GTH-II release by actions on intracellular GnRH signal transduction pathways. In goldfish, increases in $[Ca^{2+}]_i$ and PKC activation are two intracellular signalling pathways common to the action of both native GnRHs (reviewed in section I.3). In this thesis, efforts have been focused on investigating the possible effects of T on these two pathways. The influences of T on second messenger systems were examined using pituitary cells obtained from goldfish at different maturational stages. Past studies have shown that the basic composition of second messenger components leading to GTH-II release remain relatively consistent and functional throughout the different stages of the reproductive cycle in goldfish (Chang and Jobin, 1994).

IV.3.3.1. $\underline{Ca}^{2+}$

To examine the possibility that T-treatments on the goldfish pituitary cells can enhance GTH-II release by increasing the amount of GTH-II available for Ca^{2+}-induced release, the hormone response to maximally raised $[Ca^{2+}]_i$ were examined using the Ca^{2+} ionophore, ionomycin. Ionomycin has previously been shown to be effective in stimulating GTH-II release, as well as in increasing $[Ca^{2+}]_i$ in mixed population of goldfish pituitary cells and identified goldfish gonadotrophs (Chang *et al*, 1990b; Jobin and Chang, 1992a; Van Goor, 1997). In this thesis, ionomycin increased GTH-II secretion in a dose-dependent manner as described previously (Chang *et al*, 1990b). T treatment did not alter the sensitivity, potency, or the efficacy of ionomycin in stimulating GTH-II release (section III.4.2). Similarly, T treatment did not alter the sensitivity to the VSCC agonist Bay K 8644 and the GTH-II response to an effective (10 μM) Bay K 8644 dose. These observations suggest that neither the size of the Ca^{2+}-dependent GTH-II pool nor the sensitivity of the release

process to $[Ca^{2+}]_i$ increases were altered by T treatment. Thus, it is unlikely that T increased the GnRH-stimulated GTH-II release by actions at these two sites of the Ca^{2+}-dependent signalling pathway leading to GTH-II secretion. These observations in goldfish agree with results from studies on rat pituitary cells. Treatments with E_2 did not potentiate the ionophore-stimulated LH response as compared to values from untreated cells (Thomson *et al*, 1993). Thus, in rats as in teleosts, the total Ca^{2+}-dependent releasable GTH-II pool was not affected by gonadal steroid treatments. Although T does not affect the Ca^{2+}-sensitive GTH-II pool or the sensitivity of hormone secretion to $[Ca^{2+}]_i$ increases in goldfish pituitary cells, the possibility that T modulates other sites along the Ca^{2+}-dependent signalling pathway upstream from these processes cannot be entirely ruled out based on the present results.

Previous studies have shown that Ca^{2+} entry through VSCC is important for GnRH stimulation of GTH-II secretion (see section I.3.3). In addition, studies with pituitary cells from ewes showed that E_2 enhances GnRH-induced LH release by increasing Ca^{2+} currents through VSCC and GnRH-induced Ca^{2+} entry (Heyward and Clark, 1995). In rat pituitary cells, steroidal treatment also resulted in the modulation of Ca^{2+} signalling (Ortmann *et al*, 1992, 1995; see section I.7.2). Therefore, T may positively modulate the entry of extracellular Ca^{2+} through VSCC in goldfish pituitary cells. In the present study, 10 μM Bay K 8644, which has previously been shown to increase the magnitude of Ca^{2+} current flow through VSCC in identified goldfish gonadotropes by 30% (Van Goor *et al*, 1996), increased GTH-II secretion in both T-treated and non-T-treated cells (Fig. 16). This suggests that VSCC are functional in gonadotropes following T pretreatment. Perhaps by using more direct methods such as Fura-2 $[Ca^{2+}]_i$ measurements or electrophysiological studies on Ca^{2+} currents, the possibility that T treatment modulates the function of VSCC leading to an increase in the entry of extracellular calcium could be clarified. Nevertheless, a nifedipine-sensitive (VSCC-dependent) component is still present in sGnRH and

cGnRH-II action in T-treated, as in untreated cells (Figs. 17 and 18). These results indicate that T treatment did not remove the extracellular Ca^{2+}-dependence in GnRH stimulation of GTH-II release in goldfish.

Although nifedipine reduced the GTH-II responses in both T-treated and untreated cells, nifedipine did not abolish these responses (Figs. 17 and 18). There are two possible explanations. First, total inhibition of all VSCC activities by the dihydropyridine VSCC antagonist, nifedipine, is not possible. In goldfish gonadotropes, maximal (high μM) concentrations of nifedipine only caused a ≈40% reduction in the amplitude of the maximal Ca^{2+} current flowing through VSCC (Van Goor *et al*, 1996). Similarly in rat gonadotropes, only ≈50% of the extracellular Ca^{2+} entering the cell passes through dihydropyridine- and phenylalkylamine-sensitive, "L-" type VSCC (Izumi *et al*, 1989). In fact, extracellular Ca^{2+} entry through other channels, such as the "T-" type VSCC has been identified in rat gonadotropes (Stutzin *et al*, 1989). The partial dihydropyridine-sensitivity of the VSCC in goldfish gonadotropes may indicate that Ca^{2+} channels other than dihydropyridine-sensitive "L-" type channels are also involved in GnRH action on goldfish pituitary GTH-II release.

Second, GnRH-stimulated GTH-II release in goldfish pituitary does not depend solely on the entry of extracellular Ca^{2+} (for a review, see Chang *et al*, 1996 and section I.3). A recent study by Van Goor (1997) showed that GnRH can increase $[Ca^{2+}]_i$ in goldfish pituitary gonadotropes, at least in part, by mobilizing Ca^{2+} from intracellular stores. In this thesis, it is shown that T treatments preferentially enhanced the initial ("peak") phase of the GTH-II responses to sGnRH and cGnRH-II relative to the "plateau" phase response (see discussion in section IV.2.3). In rat pituitary cells, the initial "peak" phase of the LH response to GnRH depends mainly on intracellular Ca^{2+} mobilization, whereas, the plateau phase is more dependent on extracellular Ca^{2+}

entry (Catt and Stojilkovic, 1989). In goldfish pituitary cells, T pretreatment might have enhanced the ability of GnRH to mobilize Ca^{2+} from intracellular stores.

In the present study with cells prepared from sexually regressed goldfish, the inhibitory effects of nifedipine on GnRH-stimulated GTH-II release were fully reversible under normal testing conditions. In contrast, although T-treatment potentiated the GTH-II responses to GnRH even in the presence of nifedipine, the inhibitory influence of nifedipine was not reversed upon its removal in T-treated cells (washout; Figs. 17 and 18). These observations are reminescent of results from a previous study by Jobin *et al* (1996a). In this earlier report, the reversibility of nifedipine-induced inhibition of GnRH action was more evident in experiments performed with pituitary cells from sexually regressed goldfish than with cells from sexually recrudescing goldfish. In addition, the remaining GnRH-stimulated GTH-II responses during nifedipine application were higher in cells from sexually recrudescing than in sexually regressed fish. Taken together with the current observations in experiments with nifedipine, these results from Jobin *et al* (1996a) suggest that treatment of pituitary cells from sexually regressed goldfish with T converted the nifedipine-dependence properties to those characteristic of cells from recrudescent fish. How T treatment or advancing gonadal recrudescence affects the reversibility of the nifedipine-induced inhibition of GnRH stimulation of GTH-II release is not known. However, in T-treated cells, prior exposure to nifedipine alone also attenuated the response to a subsequent GnRH pulse applied following the removal of the VSCC inhibitor (Figs. 17A and 18A). Perhaps T treatment enhanced the sensitivity of the VSCC to modulation by dihydropyridine antagonists, leading to a more persistent blockade of VSCC action even following removal of nifedipine from the perifusate. This hypothesis requires further testing.

IV.3.3.2. The involvement of PKC.

Two lines of results suggest that PKC action on GTH-II release is affected by T treatment. First, the efficacy of a maximal (100 nM), as well as submaximal (0.1, 1, and 10 nM) doses of TPA in stimulating GTH-II release is enhanced by 10 nM T (Table III.4). Second, the sensitivity of GTH-II response to TPA is increased by T treatment (Table III.4). These data suggest that T treatment positively modulates PKC-stimulated GTH-II release in goldfish pituitary cells. This influence of T on the PKC-dependent GTH-II release appears to be an important mechanism by which this androgen potentiates GnRH action on GTH-II secretion. Application of the PKC inhibitor, H7, attenuated TPA-induced GTH-II secretion, and abolished both the sGnRH- and cGnRH-II-stimulated GTH-II response in T-treated cells (Figs. 14 and 15). Taken as a whole, these results strongly suggest that enhancement of PKC action on GTH-II secretion is an important factor in the action of T on potentiating GnRH-induced GTH-II responses.

The proposed involvement of PKC in T modulation of GnRH-stimulated GTH-II release is also supported by results from experiments in another model system. *In vivo* E_2 implants into ovariectomized female rats increased PKC enzyme activities in the pituitaries (Drouva *et al*, 1990). Similarly, *in vitro* E_2 treatment increases pituitary contents of phorbol-sensitive PKC isoforms in this species (Thomson *et al*, 1993). Thus, steroid (E_2) positive feedback action on GnRH-stimulated LH release in rats can also be expressed directly at the level of the gonadotropes and with PKC activation being a major target site in mediating these potentiating actions. Taken together with results from the present studies, it is also likely that T modulates the activity and/or amounts of PKC molecules in the goldfish gonadotropes to increase the magnitude of GTH-II release responses to GnRH. Previous immunoblot studies indicate that goldfish pituitary contains phorbol-sensitive α and β PKC isoforms (Jobin *et al*, 1993). Whether other isoform(s) exist in goldfish gonadotropes is at present unknown. In addition, further investigation is required to

identify the particular isoform(s) of PKC involved in T action and to determine if downregulation of PKC levels in goldfish pituitary cells through TPA-induced desensitization would affect the positive influence T on GTH-II release.

IV.4. The role of aromatization in the positive effect of T on GnRH-stimulated GTH-II release.

As indicated in the Introduction (section I.2.5.3), the action of T on GTH-II release can be exerted directly via binding to androgen receptors present in the pituitary cells (Pasmanik and Callard, 1988b). T can also act indirectly through its conversion to other metabolites by the pituitary cells. In goldfish, T can be aromatized to E_2 via aromatase enzymes present in the pituitary (Pasmanik and Callard, 1988a) or be converted to nonaromatizable metabolites such as 11-KT and DHT (Callard, 1983). In the present study, treatments with nonaromatizable androgens, 11-KT and DHT, did not mimic the ability of T to enhance sGnRH-stimulated GTH-II release (Fig. 19). This suggests that conversion of T to these nonaromatizable metabolites does not mediate T action on GnRH-induced GTH-II secretion *in vitro*. Previous *in vivo* studies with implantations of DHT and 11-KT also showed that these T metabolites were ineffective in elevating LHRH-A-stimulated GTH-II levels in the serum of female goldfish (Trudeau *et al*, 1991b). Taken together, these data strongly suggest that conversion of T to nonaromatizable androgens does not mediate the positive influence of T on GnRH action at the level of the pituitary in goldfish.

On the other hand, results from the present thesis suggest that aromatization of T to E_2 may play a role in mediating the effects of T on GnRH-stimulated GTH-II release. The application of an aromatase inhibitor, ATD, to T-treated cells abolished the potentiating effects of T on sGnRH- and cGnRH-II-stimulated GTH-II release (Fig. 20). Preliminary studies (Appendix 1) confirmed that

aromatization of exogenously applied T to E_2 by goldfish pituitary cells can occur *in vitro*. Thus, the ability of T to potentiate GnRH-induced GTH-II release probably requires its conversion, locally, to E_2. This conclusion is supported by results from a previous *in vivo* study in which ATD blocked the positive action of T implantation on LHRH-A-induced elevation in serum GTH-II levels in female goldfish (Trudeau *et al*, 1991b). In addition, *in vivo* E_2 implantations into goldfish resulted in an enhancement of sGnRH-stimulated GTH-II release from goldfish pituitary cells *in vitro*, without altering the total cellular GTH-II contents (V. Trudeau and J. P. Chang, personal communication). Hence, aromatization of T is likely an important event in the expression of the ability of T to potentiate GnRH-stimulated GTH-II release, both *in vivo* and *in vitro*. In previous *in vivo* studies (Trudeau *et al*, 1991b; discussed in Introduction section I.2.5.2), the potentiating effects of E_2 implantation on LHRH-A-induced GTH-II secretion could be easily demonstrated in sexually mature (prespawning) goldfish. In the present study, the inhibiting effects of ATD on T-induced enhancement of the GTH-II response to GnRH *in vitro* was demonstrated with pituitary cells from prespawning fish (Fig. 20). Thus, the importance of aromatization in mediating the T potentiating action on GnRH-stimulated GTH-II secretion probably exists at all times of the seasonal reproductive cycle, and is not restricted only to early stages of gonadal recrudescence. The proposed involvement of aromatization in mediating T action on GnRH stimulation of GTH-II secretion is also consistent with the potentiating influence of T on PKC-dependent hormone secretion. As E_2 has been shown to modulate PKC isoforms in rats (see section IV.3.3.2), it is possible that T, via its conversion to E_2, alters the PKC isoforms and their activity profiles, leading to increased GTH-II responses to the PKC-dependent GnRH action.

Although the direct action of T on enhancing GnRH-induced GTH-II release has been shown to involve modulation of PKC-dependent GTH-II secretion, whether other signal transduction pathways are also affected is at present unknown. In

future studies, the possible effects on AA- and cAMP-dependent exocytosis should also be investigated. As described in the Introduction (sections I.3.6 and I.3.7), AA is another signal transduction mechanism mediating sGnRH action in goldfish; whereas cAMP may be important as a positive modulator of GnRH-induced GTH-II secretion.

IV.5. Relevance of current findings to the neuroendocrine regulation of GTH-II release in goldfish.

As reviewed in the Introduction (sections I.2.5 and I.5), the positive feedback effects of gonadal steroids are important in the neuroendocrine regulation of GTH-II secretion and reproduction in teleosts as in higher vertebrates. In teleosts which reproduce on a seasonal basis, the balance between positive and negative feedback influences of gonadal steroids not only affects the onset of gonadal recrudescence and gonadal maturation, but also helps to regulate final gonadal maturation processes, ovulation and spawning. The positive regulatory actions of T on GTH-II secretion in teleosts, including the goldfish, have long been known to be mediated via modulation of the release of neuroendocrine regulators and by enhancement of long-term GTH-II synthesis (reviewed in sections I.2.5.2, I.2.5.3, I.6). In this thesis, the ability of T to positively modulate GnRH-stimulated GTH-II release by actions directly at the level of the pituitary cells has been clearly demonstrated in the goldfish. The ability of T to potentiate GTH-II responses extends to stimulatory actions of both native GnRH peptides. Results also identify this direct action of T to be functional in pituitary cells from both male and female goldfish, as well as from females alone, and regardless of the status of the seasonal gonadal reproductive condition (discussed in section IV.2). The local conversion of T to its aromatizable and nonaromatizable metabolites also appears to be an important factor in the regulation of processes leading to GTH-II synthesis in the gonadotropes. Aromatizable T metabolites, perhaps E_2, may exert a

negative influence on GTH-II protein production (discussed in section IV.3.2). These findings indicate that a direct T action at the level of the pituitary cells should be considered as an important and integral part of steroid feedback actions on the HPG axis.

The present thesis also provides information on the possible mechanisms by which T directly enhances the GnRH stimulatory influence on GTH-II secretion at the pituitary cell level (discussed in section IV.3). In particular, enhancement of the efficiency of PKC-dependent components within the GnRH signal transduction cascade has been identified as an important aspect of T positive feedback action. However, the direct positive feedback effect of T does not involve modulation of Ca^{2+}-induced GTH-II release (see section III.4.2), GnRH receptor binding (see Appendix 2) or GTH-II protein synthesis (see section III.3), which are known targets for T positive feedback action in other vertebrate models. In addition, T positive influence on GTH-II release is restricted to stimulated GTH-II secretion and does not extend to basal secretion (section IV.2.1). Thus, the direct positive feedback action of T is specific to select processes in the regulation of exocytosis. The potentiating actions of T on GnRH-induced GTH-II release also require, at least in part, its aromatization to other metabolites (section IV.4).

Experiments included in this thesis are one of a few studies to systematically compare the GTH-II release response in dispersed goldfish pituitary cells to challenges by the two native GnRHs over all three major stages of the seasonal reproductive cycle. Important differences in relative changes in the GTH-II response magnitude and kinetic profile with gonadal conditions, as well as with T treatment are identified. These findings emphasize the possible existence of differential functions of the two GnRHs in controlling GTH-II release in goldfish at different stages of gonadal recrudescence (discussed in section IV.1 and IV.2.3-5). These observations are also consistent with the established hypothesis that the two GnRHs regulate GTH-II

secretion by common, as well as dissimilar, signal transduction pathways (Chang *et al*, 1996).

Results from the present study also provide new insights into the possible importance of gonadal steroids in the neuroendocrine regulation of seasonal GTH-II secretion and reproduction in goldfish. In sexually regressed goldfish, serum levels of androgens and estrogens are low, with circulating T levels around 0.5 ng/ml (≈ 2 nM; Huggard *et al*, 1996). The relatively low level of E_2, in conjunction with a high level of 5α-reductase activity in the brain and pituitary observed in goldfish at this reproductive stage (Pasmanik and Callard, 1988a), favours the positive influence of nonaromatizable androgens on GTH-II β-subunit mRNA production and protein synthesis, while minimizing the negative effects of E_2 on these processes (discussed in section IV.3.2). As a result, pituitary GTH-II synthesis gradually increases. With the low circulating T levels, T positive feedback action preferentially enhances the GTH-II-releasing action of cGnRH-II as compared to sGnRH. (Treatment with 1 and 10 nM T enhanced GTH-II response to cGnRH-II, but not to sGnRH, in experiments with pituitary cells from sexually regressed females; Figs. 1 and 3; Tables III.1 and III.2.) At this reproductive stage, cGnRH-II may be relatively more important than sGnRH in the control of GTH-II release (discussed in section IV.1.1) and these effects of T also favour cGnRH-II action. These direct actions of T, together with other effects of gonadal steroids on increasing hypothalamic GnRH neuronal activity, contribute to a gradual increase in GTH-II release responsiveness, elevation in GTH-II secretion, and the stimulation of gonadal recrudescence.

As gonadal recrudescence proceeds, a small transient increase in pituitary and brain aromatase activity occurs in the goldfish (Pasmanik and Callard, 1988a). These changes, together with increases in both T and E_2 production in the gonads, ensures that the T:E_2 ratio remains low both within the circulation and in the local vicinity of gonadal steroid target sites. Exposure to the low nM concentrations of

T present at this reproductive stage selectively promotes the development of an enhanced sGnRH-elicited GTH-II response. With continual T exposure, the ability of sGnRH to stimulate a "biphasic" GTH-II release response characteristic of sexually recrudescent fish is acquired (Table III.1; discussed in IV.2.3). These effects, together with maintained ability of T to potentiate sGnRH- and cGnRH-II-induced GTH-II release, further increase gonadotrope responsiveness to GnRH. This elevated responsiveness is counterbalanced, in part, by the T-induced increase in the ease of GnRH-induced desensitization (Figs. 1-8; section IV.2.2). These changes, in combination with the action of T and E_2 on stimulatory and inhibitory hypothalamic influences (sections I.2 and I.5), ensure that the elevation in circulating GTH-II levels is prevented from becoming too high. The inhibitory influence of E_2 and other aromatized metabolites of T on GTH-II peptide production also exert a modulatory effect on T- and GnRH-mediated increase in long-term GTH-II gene expression and peptide synthesis. These neuroendocrine events and steroid effects all contribute to a controlled increase in serum GTH-II levels which drives continual gonadal development.

In later stages of ovarian maturation, gonadal production of E_2 decreases in favour of T and the ratio of circulating T:E_2 increases (Kagawa *et al,* 1983, 1984). Serum levels of T approach the 10-100 nM range (Huggard *et al,* 1996). With the shift in circulating T:E_2 ratio, the total negative influence of aromatized steroid metabolites on GTH-II protein synthesis is reduced. GTH-II synthetic capacity is thus at maximum. The importance of sGnRH in the neuroendocrine regulation of GTH-II release is also postulated to increase as compared to cGnRH-II at this stage (discussed in section IV.1). Despite the effects of T on decreasing the potency of the two GnRHs in stimulating GTH-II release, the efficacy of both native GnRHs is maximally enhanced by these high levels of T. The potentiating effects of T on the efficiency of post-receptor GnRH signal transduction, coupled with the increase in pituitary GnRH

and androgen receptors observed at this time of the reproductive cycle (reviewed in section I.5.1.1), result in maximal responsiveness of the gonadotrope to GnRH stimulation. The reported increase in pituitary aromatase activity observed in prespawning goldfish (reviewed in section I.5.1.1) is consistent with the requirement for aromatization in the expression of the direct positive feedback effect of T. With the appropriate external cues inducing the removal of DA inhibition and increasing GnRH neuronal activity (reviewed in section I.5.2), a rapid increase in GTH-II secretion can occur with the participation of T positive feedback. The resulting GTH-II surge causes final oocyte maturation and ovulation (Stacey *et al*, 1979). Together with other neuroendocrine mechanisms, the T-induced increase in the ease of GnRH-induced desensitization of GTH-II release plays a role in the termination of the periovulatory GTH-II surge.

As in sexually mature females, T positive feedback action may also play an important role in priming the pituitary gonadotropes for the prespawning increase in GTH-II secretion in mature males. This can be achieved by the action of T on enhancing the responsiveness of the gonadotropes to GnRH stimulation. Similarly, the increased ease of GnRH-induced desensitization likely participates in limiting the magnitude of the GTH-II surge during spawning.

In summary, results from the current study not only demonstrate the presence of direct actions of T on gonadotrope function in goldfish, they also contribute to the understanding of the complex events involved in the neuroendocrine regulation of GTH-II release, synthesis, and reproduction in this teleost species.

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VI. *Appendix 1: Aromatization of T by Goldfish Pituitary Cells.*

VI.1. Introduction.

In experiments reported in this thesis, dispersed goldfish pituitary cells were cultured overnight in serum-containing medium and T-supplemented medium prior to testing. The presence of aromatase activities in the goldfish pituitary has been reported (Pasmanik and Callard, 1988). Thus, the pituitary cells may be able to aromatize androgens to E_2 *in vitro*. In this preliminary study, the ability of goldfish pituitary cells to aromatize the T present in serum-containing medium or as added steroid supplements during an overnight incubation was examined.

VI.2. Materials and methods.

Pituitaries were obtained from sexually regressed male and female goldfish. Cells were dispersed and cultured as previously described in section II.3. Culture dishes contained either 2 million cells or no cells. In dishes containing cells, 200 µl of medium containing either charcoal-stripped horse serum, horse serum, or horse serum in combination with 0.01, 0.1, or 1 µM T were added. In dishes containing no cells, plating medium alone, or plating medium containing charcoal-stripped horse serum in combination with 0, 0.01, 0.1, or 1 µM T were added. Treatments were performed in duplicate. Following a 16-h overnight incubation, medium from each culture dish was removed and frozen immediately for subsequent measurement of T and E_2. T and E_2 levels were determined by RIA using the methods described by Van Der Kraak and Chang (1990) and Van Der Kraak *et al* (1990), respectively. Average values are reported. No statistical analysis was performed. Assay sensitivities were approximately 3 pg per tube.

VI.3. Results and discussion.

The plating medium used contained small, but detectable, amounts of T and E_2. Addition of either normal or charcoal-stripped horse serum did not cause a large difference in T or E_2 levels in the media (Table V.1.). These concentrations of T (between 0.1 and 0.2 ng/ml) and E_2 (between 0.2 and 0.3 ng/ml) can be considered as the basal steroid levels in the plating medium. Application of T to the plating medium caused a dose-dependent increase in T levels and a small increase in E_2 levels in dishes containing no cells in the media (Table V.1.). The same treatment to dishes with cells also caused a dose-dependent increase in T levels, but the concentrations of T in the 1 μM and 0.01 μM T-treated groups were lower than the corresponding values in dishes not containing cells. On the other hand, the levels of E_2 in the T-treated groups were generally higher in dishes containing cells than in dishes without cells. This suggests that goldfish pituitary cells can metabolize exogenously added T *in vitro* and that some of the androgens may be converted to E_2. These preliminary results indicate the possible aromatization of T to E_2 within the goldfish pituitary cells *in vitro*.

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Table VI.1. Levels of T and E_2 in medium following overnight incubation in the presence or absence of dispersed goldfish pituitary cells. Results presented are averages of replicate treatments.

Presence of pituitary cells (2 million cells)	Incubation condition	T content (ng/ml)	E_2 content (ng/ml)
cells	plating medium	0.10	0.26
cells	medium+horse serum (HS)	0.10	0.23
cells	medium+HS (stripped)	0.10	0.28
cells	medium+HS+1 µM T	207.80	0.62
cells	medium+HS+0.1 µM T	17.38	0.56
cells	medium+HS+0.01 µM T	1.73	0.39
no cells	plating medium	0.18	0.23
no cells	medium+cytodex beads	0.12	0.28
no cells	medium+beads+HS	0.14	0.28
no cells	medium+HS(stripped)+1 µM T	259.30	0.47
no cells	medium+ HS(stripped)+0.1 µM T	14.70	0.46
no cells	medium+HS(stripped)+0.01 µM T	2.77	0.29

VII. *Appendix 2: Effects of T on GnRH Receptor Binding in Dispersed Goldfish Pituitary Cells.*

VII.1. Introduction.

In goldfish, pretreatment of pituitary cells with T was shown to potentiate GnRH-stimulated GTH-II release (this thesis). This effect occurs directly at the level of the pituitary cells. One of the many possible mechanisms by which T could enhance GnRH-stimulated GTH-II release is by modulating GnRH binding to its receptors. Earlier studies by Trudeau *et al.* (1993) showed that *in vivo* implantations of T-containing capsules into goldfish for 5 days did not cause changes in the binding affinity or capacity of both the high- and low-affinity pituitary GnRH binding sites. In this preliminary study, the effects of *in vitro* exposure to T on pituitary GnRH binding was examined. Dispersed goldfish pituitary cells were treated with T and the binding capacity for cGnRH-II was estimated.

VII.2. Materials and methods.

Tris[hydroxymethyl]aminomethane (Tris) was purchased from Sigma. cGnRH-II was from Peninsula Laboratories. cGnRH-II was labelled with ^{125}I-Na by Dr. P. Rosenblum using procedures in Rosenblum *et al* (1994). GnRH radioreceptor binding assays were performed according to a procedure modified from Habibi *et al* (1987).

Briefly, pituitaries were obtained from postspawning male and female goldfish and dispersed pituitary cells prepared as described in section II.3. Each culture dish contained 1.8 million cells. The cells were cultured for 24 h with either 0 or 10 nM

T in 1 ml plating medium. Cell suspensions were then transferred to 1.5 ml polypropylene microcentrifuge tubes, and the cell pellets were collected by centrifugation (200 x g, 10 min). Cell pellets were then resuspended in 100 µl of ice-cold assay buffer (10 mM Tris-HCl containing 1 mM dithiotheritol and 0.5 % BSA, pH=7.6). All incubations (in triplicates) were carried out in polypropylene microcentrifuge tubes (500 µl volume) precoated by overnight exposure to Tris-HCl buffer containing 2.5% BSA. 100 µl of ^{125}I-cGnRH-II (≈30,000-40,000 cpm/tube) was added to microcentrifuge tubes containing the cell suspension. Non-specific binding (NSB) was determined in the presence of 10 µM cGnRH-II. All tubes were incubated on ice for 2 h. After the incubation period, cell pellets were collected by centrifugation (10,000 x g, 1 min) and the supernatant removed by aspiration. Cell pellets were quickly washed with 200 µl ice-cold assay buffer. Cell pellets were again collected by centrifugation. The bottom part of each centrifuge tube containing the cell pellet was cut and transferred into a 12 x 75 mm glass tube for counting in a gamma counter. Total and specific GnRH binding were calculated.

VII.3. Results and discussion.

Results from 2 replicate experiments were pooled. Control cells had an average specific ^{125}I-cGnRH-II binding of 9.72%. In contrast, the specific binding in T-treated group was only 1.33% (Table 1). This suggests that T treatment of goldfish pituitary cells not only did not increase GnRH receptor binding capacity, but on the contrary, lowered the GnRH receptor binding capacity. It is unlikely that the potentiating effect of T on GnRH-stimulated GTH-II release is due to an increase in GnRH receptor binding capacity. Whether T-treatment affects GnRH binding affinity was not determined in these preliminary experiments.

VII.4. References.

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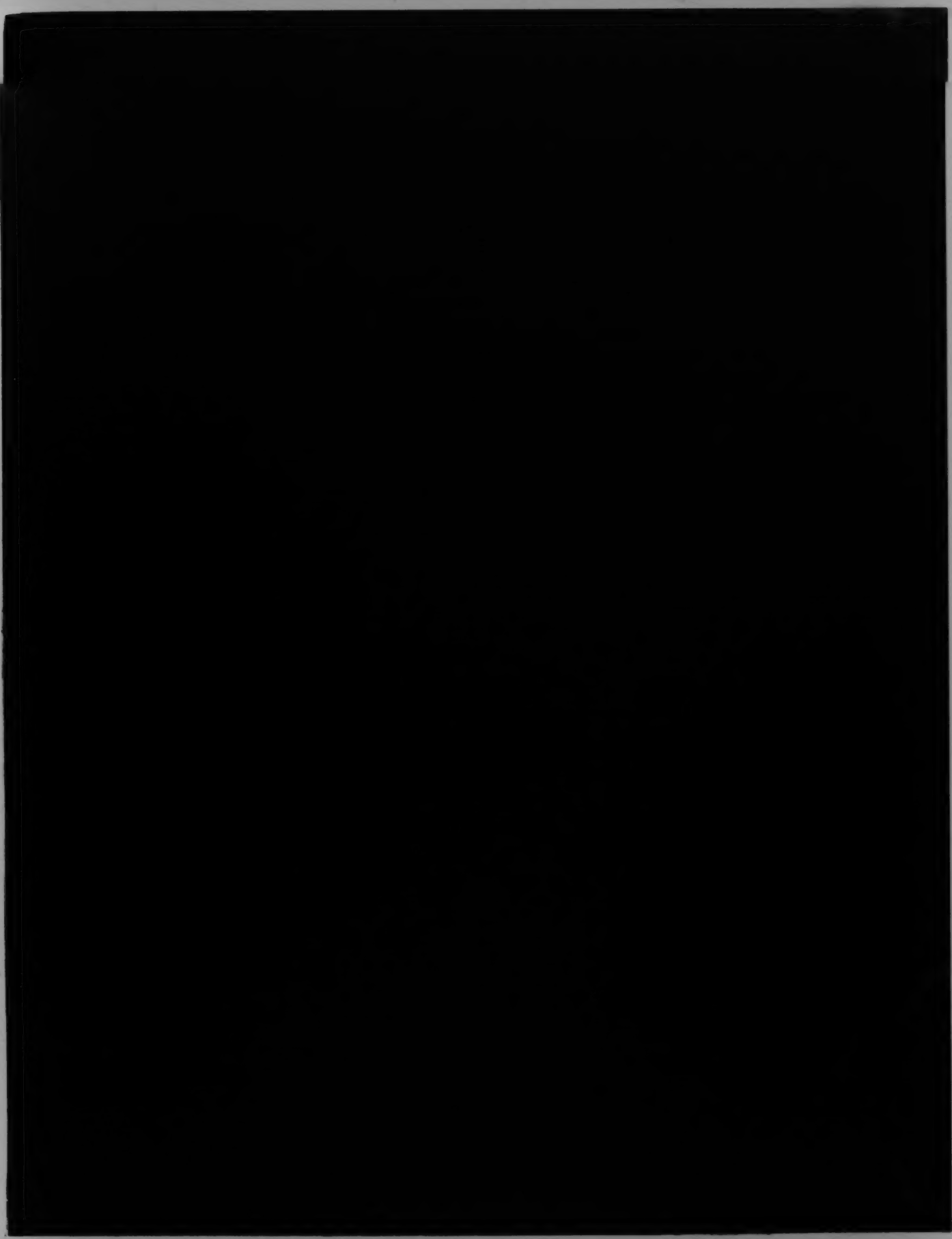
Table VII.1. Effects of 24-h pretreatment with 10 nM T on GnRH receptor binding capacity in dispersed pituitary cells prepared from postspawning goldfish.[1]

Treatment	Total Count (cpm)	Total Bound (cpm)	Non-specific Binding (NSB, cpm)	Specific Bound (%)[2]
Control	38838 ± 1077	3987 ± 318	600 ± 65	9.72
Testosterone	38838 ± 1077	1392 ± 88	893 ± 47	1.33

[1] Values reported are mean ± SEM (n = 6).

[2] Specific Bound = (Total Bound - NSB) / (Total Count - NSB) x 100%.